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(54) Title: IDENTIFICATION OF EUKARYOTIC SPECIES USING PROBES DERIVED FROM THE INTERGENIC REGION OF PAIRS OF DIVERGENTLY TRANSCRIBED HISTONE GENES

(57) **Abstract:** A method for detecting a eukaryotic species in a sample. The method comprises the steps of contacting a first primer complementary to the coding sequence of a first histone gene of a pair of divergently transcribed histone genes located in the sample and amplifying at least a segment of a non-coding sequence located between and contiguous with the coding sequence of the first and a second histone gene of the pair to produce a probe that is substantially complementary to the non-coding sequence. The probe is compared with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species thereby detecting whether said species is present in said sample.

Identification of eukaryotic species using probes derived from the intergenic region of pairs of divergently transcribed histone genes

FIELD OF THE INVENTION

The invention relates to methods and kits for the identification of species of eukaryotic organisms, and in particular, to methods for the identification of species of eukaryotic organisms using probes produced from histone genes.

BACKGROUND OF THE INVENTION

Eukaryotic organisms constitute a substantial portion of living cells, and play important roles in areas such as food production, industrial processing and human and animal health.

Many species of eukaryotic organisms are used widely in manufacture of many products. A variety of industrial processes such as brewing, wine making, distilling and other fermentation-based processes, use various species of yeast and/or filamentous fungi. For example, *Saccharomyces cerevisiae* is used widely in the manufacture of bread and wine. Fungal species such as, for example, *Aspergillus* sp. are used in the production of enzymes, proteins and chemicals for use in the pharmaceutical industry and food and industrial processes.

Many species of eukaryotic organisms also contaminate many products. In a variety of industrial processes such as the production of yoghurt, fruit juices and other food and beverage (alcoholic and non-alcoholic) products, yeast or filamentous fungi are contaminants. For example in beer and wine manufacture, yeasts other than the specialised strain of *Saccharomyces* added by the brewer or wine maker are termed wild yeasts. A wide range of wild yeast cause adverse effects on wine quality such as, for example, *Brettanomyces anomalous* etc.

In human and animal health, certain species of eukaryotic organisms are also important pathogens such as, for example, *Candida albicans*, *Aspergillus* sp., *Toxoplasma gondii*, *Leishmania* sp., *Cryptospiridium* sp., and *Giardia* sp. etc.

Consequently, it is useful to be able to identify the species that are present in a sample that is obtained, for example, from a manufacturing process or a pathology sample. Moreover, given that potential spoilage organisms such as yeast are a problem in a variety of industrial processes such as brewing, wine making, distilling and other fermentation-based processes (G Reed and T.W. Nagodawithana, 1991, yeast technology, 2nd edition Van Nostrand Reinhold: New York), the rapid identification of such organisms is important. In human and animal health, rapid detection of the presence of certain organisms may also be critical in determining treatment regimes. Thus, the ability to uniquely identify eukaryotic organisms from a sample has many applications.

In many cases, however, eukaryotic organisms are present in a milieu of cells of different species, and identification of one or more organisms present in a sample is problematic. This is further compounded by the need in many cases to culture or isolate the organism prior to identification.

To avoid the need to isolate or culture organisms, a number of nucleic acid based tests have been developed to detect the presence of certain organisms. In particular, since the advent of the polymerase chain reaction (PCR) and other nucleic acid amplification techniques, the impact of DNA probe technology in the identification of biological material has increased. One form of nucleic acid based test described in the literature and commercially available uses the transcribed intergenic spacer regions (ITS) between ribosomal RNA genes for the identification of organisms (R. Rossau and H. Van Heuverswyn). Hybridisation probes derived from the spacer

region between 16S and 23S rRNA genes are used for the detection of non-viral microorganisms (US Patent no. 5,945,282). However, while the DNA probe approach is specific and sensitive, and may therefore replace, or at least supplement conventional identification techniques with time, there are some limiting factors. For example, although the ITS sequence is found in all cellular organisms and has been exploited, non-specific cross hybridisation has been a problem, even with carefully designed probes.

What is needed are nucleic acid probes and methods for identifying species of eukaryotic organisms which can be used as an alternative to, or in conjunction with, the ITS sequence. As a consequence, the inventor has developed nucleic acid probes and methods of identifying eukaryotic organisms which requires no, or at least limited, isolation of the organisms and does not rely on the ITS sequence.

In eukaryotic organisms, the histone genes are essential genes that are amongst the most highly conserved genes in the eukaryotic genome. The amino acid sequence of each histone gene of the eukaryotic organism is highly conserved not only within the same species of organism but between different species and genus of organism. Because of this conservation in the coding sequence of the histone genes, the histone genes were not considered useful for identifying species of eukaryotic organisms because it was considered that probes generated from the histone genes would cross-hybridize with many different species.

The inventor has surprisingly found that while the nucleic acid sequence encoding the histone protein of divergently transcribed histone genes (coding sequence) is highly conserved, the non-coding sequence located between the coding sequence of each pair of divergently transcribed histone genes is highly variable between different species. The inventor has found that this variable region of the histone genes may be used as a

probe to detect and/or identify eukaryotic organisms belonging to a particular species. The inventor has further observed that the histone genes are organised differently in different eukaryotes. In particular the inventor has found that lower eukaryotes such as yeasts, mycelial fungi and protozoans have both H2a, H2b histone gene pairs and H3, H4 histone gene pairs organised around divergent promoters whereas the higher eukaryotes, such as humans have different organisations of the four genes. For example, neither humans nor higher plants have divergently arranged H3 H4 genes. The inventor has realised that the H3 H4 pair of lower eukaryotic organisms can be selectively amplified even in the presence of a large excess of tissue from either higher animals or plants.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides a method for detecting a eukaryotic species in a sample comprising the steps of:

(i) contacting a first primer complementary to the coding sequence of a first histone gene of a pair of divergently transcribed histone genes located in said sample and amplifying at least a segment of a non-coding sequence located between and contiguous with the coding sequence of the first and a second histone gene of the pair to produce a probe substantially complementary to said non-coding sequence; and

(ii) comparing the probe with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species thereby detecting whether said species is present in said sample.

Preferably, the probe is compared with the one or more reference nucleic acids by hybridising the probe with the one or more reference nucleic acid molecules under stringent hybridisation conditions.

Preferably, the method further comprises the step of providing a second primer complementary to the sequence of the second strand of said coding sequence of the second histone gene, and contacting said second primer with said nucleic acid molecules.

Preferably, the method further comprises a step before step i) of extracting nucleic acid from one or more eukaryotic species in said sample.

Preferably, the first histone gene coding sequence comprises at least 15bp and the second histone gene coding sequence comprises at least 15bp.

The pair of histone genes may be any pair of histone genes provided the genes are divergently transcribed and the coding region of each gene is separated by a non-coding region. Preferably, the pair of divergently transcribed histone genes is the H2a and H2b histone gene pair, or the H3 and H4 histone gene pair.
Preferably, the first histone gene is selected from the group comprising H2a, H2b, H3 and H4. The second histone gene will be the remaining histone gene of the pair, or in other words, the histone gene of the pair that is not the first histone gene. For example, when the first histone gene is H2a, the second histone gene will be H2b, when the first histone gene is H2b, the second histone gene will be H2a, when the first histone gene is H3, the second histone gene is H4, and when the first histone gene is H4, the second histone gene is H3.

The probe may be produced by amplifying at least a segment of the non-coding sequence utilising any methods known in the art for primer based amplification of nucleic acid. In one embodiment, the segment of non-coding sequence is amplified by extending the first primer. Preferably, the first primer is extended using an RNA or DNA polymerase.

In another embodiment, the segment of non-coding sequence is amplified by extending the first and second primer. Preferably, the segment of non-coding sequence is

amplified by extending the first and second primers in a polymerase chain reaction (PCR).

The sequence of the probe may be complementary to the entire non-coding sequence between the coding sequences of the first and second histone genes, or to any portion of this sequence. Preferably, the sequence of the probe comprises at least 15 contiguous nucleotides complementary to the non-coding sequence. More preferably, the sequence of the probe comprises at least 15 contiguous nucleotides complementary to at least one promoter of the pair of histone genes. Even more preferably, the sequence of the probe comprises at least 15 contiguous nucleotides complementary to the sequence corresponding to both promoters of the histone genes. Most preferably, the sequence of the probe is substantially complementary to the entire non-coding sequence between the coding sequence of the first and second histone genes of an organism of the sample.

The sequence of the probe when produced from a particular species of eukaryotic organism in the sample, will be specific to that species and may be used to identify the species of eukaryotic organism in the sample. It is envisaged that the probe can be produced from any species of eukaryotic organism provided the species possesses at least one pair of divergently transcribed histone genes. Eukaryotic organisms that may be identified using the method of the invention include, for example, protozoans, algae, cnidarians, annelids, nematodes, ciliates, yeast and mycelial fungi having at least one pair of divergently transcribed histone genes.

Preferably, the eukaryotic species is a species of yeast or mycelial fungus. Species which may be identified using the method of the present invention include, for example, *Alternaria alternata, Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus versicolor, Blastomyces dermatiditis, Candida albicans, Candida dubliniensis, Candida krusei,*

Candida norvegensis, Candida parapsilosis, Candida tropicalis, Candida glabrata, Coccidioides immitis, Cryptococcus neoformans, Epidermophyton floccosum, Histoplasma capsulatum, Malassezia furfur, Microsporum canis, Mucor spp., Paracoccidioides brasiliensis, Penicillium marneffei, Pityrosporum ovale, Pneumocystis carinii, Sporothrix schenkii, Trichophyton rubrum, Trichophyton interdigitale, Trichosporon beigelii, Brettanomyces clausenii, Brettanomyces custerii, Brettanomyces anomalous, Brettanomyces naardenensis, Candida himilis, Candida lusitaniae, Candida multigemmis, Candida utilis, Candida intermedia, Candida saki, Candida solani, Candida tropicalus, Candida versatilis, Candida bechii, Candida famata, Candida lipolytica, Candida stellata, Candida vini, Debaromyces hansenii, Dekkera, intermedia, Dekkera anomala, Dekkera bruxellensis, Endomyces fibuliger, Geotrichium candidum, Hansenula fabiani, Hanseniaspora uvarum, Hansenula anomala, Hanseniaspora guillermondii Hanseniaspora vinae, Hypopichia burtonii, Kluyveromyces lactis, Kloeckera apiculata, Kluyveromyces marxianus, Kluyveromyces fragilis, Leucosproidium scottii, Metschnikowia pulcherrima, Pichia anomala, Pichia guilliermodii, Pichia orientalis, Pichia fermentans, Pichia memranefaciens, Rhodotorula glutinis, Rhodotorula mucilaginosa, Rhodotorula rubra, Saccharomyces bayanus, Saccharomyces cerevisiae, Saccharomyces dairiensis Saccharomyces exigus, Saccharomyces uinsporus, Saccharomyces uvarum, Saccharomyces oleaginosus, Saccharomyces boulardii, Saccharomycodies ludwigii, Schizosaccharomyces pombe, Torulaspora delbrueckii, Torulopsis holmii, Torulopsis stellata, Yarrowia lipolytica, Zygoaccharomyces baili, Zygosaccharomyces bisporus and *Zygosaccharomyces rouxii.*

Examples of protozoan organisms that may be detected using the method of the invention includes *Trichomonas* sp.

Preferably, the first primer is complementary to the first strand of the coding sequence of the first histone

gene of a plurality of species of eukaryotic organisms. For example, the first primer may be complementary to the first strand of the coding sequence of a histone gene of one or more of the above mentioned eukaryotic organisms.

Preferably, the second primer is complementary to the second strand of the coding sequence of the second histone gene of a plurality of species of eukaryotic organisms. For example, the second primer may be complementary to the second strand of the coding sequence of the second histone gene of one or more of the above mentioned eukaryotic organisms.

In one embodiment, the first primer is complementary to the first strand of the coding sequence that encodes the H2a protein. Preferably, the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence VGAGAPVYLTAVLEY (SEQ ID NO. 1 or GNVTIAQGGVLPN (SEQ ID NO. 3). More preferably, the amino acid sequence is GAPVYLT (SEQ ID NO. 2), QGGVVPN (SEQ ID NO. 4) or APVYLTAAV (SEQ ID NO. 5).

In another embodiment, the first primer is complementary to the first strand of the coding sequence that encodes the H2b protein. Preferably, the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence VLKQTHPDTG (SEQ ID NO. 6) or QTAVRLILPGELAKH (SEQ ID NO. 8). More preferably, the amino acid sequence is QTHPDTG (SEQ ID NO. 7) or PGELAKH (SEQ ID NO. 9).

In another embodiment, the first primer is complementary to the first strand of the coding sequence which encodes the H3 protein. Preferably, the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of MARTKQTA (SEQ ID NO. 10), PGTVALRE (SEQ ID NO. 11), ALREIRRYQ (SEQ ID NO. 12), GGVKKPHRY (SEQ ID NO. 13) and GGKAPRKQ (SEQ ID NO. 14). In another embodiment, the first primer is complementary

to the first strand of the coding sequence which encodes the H4 protein. Preferably, the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of GITKPAIRR (SEQ ID NO. 15), GKGGKGLGKGG (SEQ ID NO. 16), GKGGAKRHR (SEQ ID NO. 17), MSGGKSGGK (SEQ ID NO. 18) and QGITKPAIRR (SEQ ID NO. 19).

In embodiments where the first primer is complementary to the first strand of the coding sequence that encodes the H2a protein, then the second primer may be complementary to the second strand of the coding sequence that encodes the H2b protein. Preferably, the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence VLKQTHPDTG (SEQ ID NO. 6) or QTAVRLILPGELAKH (SEQ ID NO. 8). More preferably, the amino acid sequence is QTHPDTG (SEQ ID NO. 7) or PGELAKH (SEQ ID NO. 9).

In embodiments where the first primer is complementary to the first strand of the coding sequence that encodes the H2b protein, then the second primer may be complementary to the second strand of the coding sequence that encodes the H2a protein. Preferably, the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence VGAGAPVYLTAVLEY (SEQ ID NO. 1 or GNVTIAQGGVLPN (SEQ ID NO. 3). More preferably, the amino acid sequence is GAPVYLT (SEQ ID NO. 2), QGGVVPN (SEQ ID NO. 4) or APVYLTAAV (SEQ ID NO. 5).

In embodiments where the first primer is complementary to the first strand of the coding sequence that encodes the H3 protein, then the second primer may complementary to the second strand of the coding sequence that encodes the H4 protein. Preferably, the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of GITKPAIRR (SEQ ID NO. 15), GKGGKGLGKGG (SEQ ID NO. 16), GKGGAKRHR (SEQ ID NO. 17),

MSGGKSGGK (SEQ ID NO. 18) and QGITKPAIRR (SEQ ID NO. 19).

In embodiments where the first primer is complementary to the first strand of the coding sequence that encodes the H4 protein, then the second primer may be complementary to the second strand of the coding sequence that encodes the H3 protein. Preferably, the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of MARTKQTA (SEQ ID NO. 10), PGTVALRE (SEQ ID NO. 11), ALREIRRYQ (SEQ ID NO. 12), GGVKKPHRY (SEQ ID NO. 13) and GGKAPRKQ (SEQ ID NO. 14).

The sequence of the first and/or second primer may be degenerate or non-degenerate. Preferably, the sequence of the primer is degenerate. Examples of degenerate primer sequences that are complementary to the coding sequence of the first or second histone genes are as follows:

H2BR	CCAGTGTCNGGRTGNRYYTG	(SEQ ID NO. 20)
H2AR	GTTAGATANACNGGNGCNCC	(SEQ ID NO. 21)
YeastH3ID	CTTGCAGTTTGYTTRGTDCKNGCC	(SEQ ID NO. 22)
H2R1	GYTARRTAARCKGGAGCACC	(SEQ ID NO. 23)
YeastH4ID	CCTTTTCCACCTTTWCCNCTDCCRG	(SEQ ID NO. 24)
H2B1	CCAGTATCAGGRTGNACNTG	(SEQ ID NO. 25)
H2AR1	GYTARRTAARCKGGAGCACC	(SEQ ID NO. 26)
H2BRYEAST	CCAGTGTCAGGYTGNGTYTG	(SEQ ID NO. 27)
H4FIL	AATAGCGGGYTTNGTDATNCCYTG	(SEQ ID NO. 28)
H3P1	GGCNMGNACTAANCAAACAGCTAG	(SEQ ID NO. 29)
H2Bfil	GCNCTNCGNGARATTCGTCGCTAC	(SEQ ID NO. 30)
H3P3	CCNGGNACNGTNGCCTTGAGAG	(SEQ ID NO. 31)
H3P4	GARGAYACYAAYYTNKGCGCTATCCAC	(SEQ ID NO. 32)
H3P5	GGNGGNGTNAARAAGCCYCAYAG	(SEQ ID NO. 33)
H3P6	GGNGGNAARGCNCCRAGAAAGCAACTAGC	(SEQ ID NO. 34)
H3R1YEAS	CTTTCTTGCTGTYTGYTTNGTYCTNGC	(SEQ ID NO. 35)
H2AFIL	GCNCCNGTNTAYCTCGCTGCTGTT	(SEQ ID NO. 36)
H3R2YEAS	CTCTCAAGGCNACNGTNCCNGG	(SEQ ID NO. 37)
H3FIL	GTAGCGACGAATYTCNCGNAGNGC	(SEQ ID NO. 38)
H4P1	GGNATNACNAARCCRGCTATCAGTCGG	(SEQ ID NO. 39)

H4P2	GGNAARGGNGGNAARGGTCTGGGAAAAGGTGG	(SEQ ID NO. 40)
H4P3	GGNAARGGNGGNGCYAAACGTCATAG	(SEQ ID NO. 41)
H4P4	GAYAAYATHCARGGGATCACGAAG	(SEQ ID NO. 42)
H4P5	CARGGNATHACRAARCCRGCTATCCGACG	(SEQ ID NO. 43)
H4R1YEAS	GATAGCTGGYTTNGTNATNCC	(SEQ ID NO. 44)
H4R1FIL	GATAGCTGGYTTNGTNATNCC	(SEQ ID NO. 45)

Wherein the nucleotides are represented as follows: A is adenine, T is thymine, C is cytosine, G is guanine, R is adenine or guanine, Y is cytosine or thymine, K is guanine or thymine, M is adenine or cytosine, W is adenine or thymine, H is adenine, cytosine or thymine, B is guanine, thymine or cytosine, D is guanine, adenine or thymine, N is adenine, guanine, cytosine or thymine.
For example, the first primers for amplifying the non-coding sequence located between the coding sequence of histone genes H2A and H2B may include any one of SEQ ID NOS 20, 24, 25, 27 and 28 and suitable second primers may include any one of SEQ ID NOS 21, 22, 23 and 26. The first primers for amplifying the non-coding sequence located between the coding sequence of histone genes H3 and H4 may include any one of SEQ ID NOS 29 to 38, and suitable second primers may include any one of SEQ ID NOS 39 to 45.

The non-coding sequence may be amplified using non-degenerate primers. Examples of non-degenerate primer sequences that are complementary to the coding sequence of the first or second histone genes are as follows:

H2Acere	GTTAGATAGACTGGAGCACC	(SEQ ID NO. 46)
H2BRCere	CCTGTGTCTGGGTGAGTTTG	(SEQ ID NO. 47)
H3R1CERE	CTTTCTTGCTGTTTGCTTTGTTCTGGC	(SEQ ID NO. 48)
H3R2CERE	CTCDTCAAGGCAACAGTACCTGG	(SEQ ID NO. 49)

H4R1CERE GATAGCTGGCTTAGTGATACC (SEQ ID NO. 50)

The one or more reference nucleic acid molecules of the method of the present invention may be any nucleic acid molecule which comprises sequence that is complementary to the non-coding sequence between the coding sequence of a pair of divergently transcribed histone genes of a eukaryotic species and can therefore hybridise to the non-coding sequence to thereby indicate whether the eukaryotic species is present in the sample. For example, the reference nucleic acid molecules may be a primer that is complementary to the non-coding sequence, isolated portions of nucleic acid sequence located in the non-coding sequence such as oligonucleotide sequences, larger nucleic acid fragments such as previously isolated nucleic acid probes of the first aspect, or genomic DNA from a eukaryotic species.

In one embodiment, the method permits identification of a eukaryotic species by incubating one or more probes with one or more reference nucleic acid molecules from a plurality of eukaryotic species under conditions which permit hybridisation of substantially complementary sequences. The reference nucleic acid may be from any eukaryotic species which possesses a pair of divergently transcribed histone genes. The reference nucleic acid may be from, for example, one or more of the eukaryotic organisms mentioned above. The source of the reference nucleic acid may vary depending on the sample and requirements of the method. The reference nucleic acid may be obtained from, for example, any species of yeast, including for example, one or more of the yeast species mentioned above.

The reference nucleic acid may be obtained from any species of fungus. The species of mycelial fungus may include fungal pathogens such as, for example, one or more of the species of fungus mentioned above.

In one embodiment, the one or more reference nucleic acid may be a panel of reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species. The panel of reference nucleic acid molecules may be in any form which permits hybridization with the nucleic acid probe. Preferably, the panel of reference nucleic acids is immobilised on a solid support such as, for example, a membrane such as nylon or nitrocellulose, on a glass slide or microchip.

Nucleic acid probes produced in the method of the invention may be used subsequently as a panel of reference nucleic acid to identify eukaryotic species.

In another embodiment, the one or more reference nucleic acid molecules is a first reference primer complementary to a first strand of the non-coding sequence of one or more eukaryotic species and the method further comprises the steps of:

(i) extending the first reference primer that is hybridised to the first strand of the non-coding sequence to amplify at least a segment of said non-coding sequence; and

(ii) detecting the amplified segment to thereby detect whether the eukaryotic species is present in sample. Optionally, the method further comprises the step of providing a second reference primer complementary to the sequence of the second strand of said non-coding sequence.

In a second aspect, the invention provides a kit for detecting the presence of one or more species of eukaryotic organisms, comprising:

(i) at least a first primer complimentary to the sequence of the first strand of the coding sequence of a first histone gene for amplifying at least a segment of the non-coding sequence to produce a probe substantially complementary to the non-coding sequence between the coding sequence of the first and second histone gene, and

(ii) instructions for use.

Preferably, the kit further comprises a second primer complementary to the sequence of the second strand of the coding sequence of the second histone gene. The kit may further comprise one or more reference nucleic acid sequences comprising the non-coding sequences of one or more eukaryotic species.

In a third aspect, the invention provides a nucleic acid molecule produced by a method according to the first aspect.

In a fourth aspect, the invention provides a probe for identifying eukaryotic species comprising a nucleic acid molecule according to the third aspect, together with a detectable moiety.

In a fifth aspect, the invention provides a primer for producing a nucleic acid molecule according to third or fourth aspect.

In a sixth aspect, the invention provides a kit for detecting a eukaryotic species, comprising a nucleic acid molecule according to the third or fourth aspects.

In a seventh aspect, the invention provides a kit for detecting a eukaryotic species comprising a probe according to the fourth aspect.

In an eighth aspect, the invention provides a kit for detecting a eukaryotic species comprising a primer according to the fifth aspect. Preferably, the primer is selected from the group consisting of SEQ ID NO. 153 to 234.

In a ninth aspect, the invention provides a method for detecting a eukaryotic species in a sample comprising the steps of:

(i) extracting one or more nucleic acid molecules from the sample, wherein the nucleic acid molecules comprise the non-coding sequence located between and contiguous with the coding sequence of a pair of divergently transcribed histone genes; and

(ii) hybridising the non-coding sequence from the sample with the non-coding sequence of one or more

reference nucleic acids thereby detecting the eukaryotic species.

Preferably, the reference nucleic acid is a nucleic acid probe comprising a sequence selected from one or more of SEQ ID NO: 153 to 234.

In one embodiment of the ninth aspect, the reference nucleic acid is a first reference primer complementary to a first strand of the non-coding sequence of one or more eukaryotic species and the method comprises the further steps of:

(iii) extending the first reference primer that is hybridised to the first strand of the non-coding sequence to amplify at least a segment of said non-coding sequence; and

(iv) detecting the amplified segment thereby detecting whether species is present in sample.

Optionally, the method further comprising the step of contacting a second reference primer complementary to the sequence of the second strand of said non-coding sequence.

Any of the kit for use in the method of the invention may further comprise, for example, solutions, buffers, enzymes and co-factors for preparing the sample for amplification with the primers of the kit; solutions, buffers, enzymes and co-factors for amplifying nucleic acid from the sample using the primers; solutions, buffers, enzymes and co-factors for incubating the amplified nucleic acid with the reference nucleic acid; instructions for use of the kit.

In a tenth aspect, the invention provides a method for detecting a eukaryotic species in a clinical sample comprising the steps of:

(a) contacting a first primer complementary to the coding sequence of a H3 histone gene located in said sample and a second primer complementary to the coding sequence of a H4 histone gene located in said sample and amplifying at least a segment of a non-coding sequence located between and

contiguous with the coding sequence of the H3 and H4 histone genes to produce a probe substantially complementary to the non-coding sequence;

(b) comparing the probe with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species thereby detecting whether the species is present in the clinical sample.

Preferably, the probe is compared with the one or more reference nucleic acids by hybridising the probe with the one or more reference nucleic acid molecules under stringent hybridisation conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an alignment of nucleic acid sequence amplified from between the translation start site of the H2a and H2b gene of *Saccharomyces cerevisiae, Zygosaccharomyces rouxii* and *Torulaspora delbrueckii, illustrating the lack of homology within the noncoding region between yeast of different genera.*

Figure 2 shows a result using an embodiment of the method of the invention illustrating species specific amplification of the non-coding sequence from *Saccharomyces cerevisiae, Zygosaccharomyces rouxii* or *Kluyveromyces marxianus* cells (as indicated on the left hand side of the figure) using primers specific to the non-coding sequence of *Saccharomyces cerevisiae, Zygosaccharomyces rouxii* or *Kluyveromyces marxianus* (as indicated above each well). Darkened wells indicate wells in which the non-coding sequence has been amplified.

Figure 3 shows the result of gel electrophoresis of the PCR product from reactions in which the non-coding region between the H3 and H4 histone genes has been amplified from 38 different species of fungus using degenerate primers.

Figure 4 shows the result of gel electrophoresis of the PCR product from reactions in which the non-coding region between the H3 and H4 histone genes has been amplified from *Aspergillus ochraecus, Aspergillus fumigatus, Penicillium crustosum, Rhizopus stolonifer.*

Figure 5 shows the result of electrophoresis of PCR product from reactions in which the non-coding region between the H2a and H2b histone genes from various yeasts has been amplified using degenerate primers.

Figure 6 shows the result of electrophoresis of PCR products illustrating selective amplification of the non-coding region between the H3 and H4 histone gene pair of yeast from a sample containing a mixture of human cells and yeast.

Figure 7 shows the result of electrophoresis of PCR products resulting from reactions in which the non-coding region between the H3 and H4 histone gene pairs has been amplified and subsequently digested with HaeIII.

Figure 8 illustrates the sequence obtained from sequencing the non-coding region between the H2a and H2b and H3 and H4 histone genes of *Saccharomyces cerevisiae*.

Figure 9a shows the result of electrophoresis of the PCR product resulting from amplification of the non-coding region between the H3 and H4 histone gene pair of yeast from a sample containing yeast and human cells using degenerate primers. Figure 9b illustrates the result of electrophoresis of the PCR product generated from amplification of the PCR product obtained in Figure 9a with a pair of primers complementary to the non-coding sequence between the H3 and H4 histone genes.

Figure 10a shows the result of electrophoresis of the PCR product resulting from amplifying the non-coding region between the H3 and H4 histone gene pair of various fungal species using degenerate primers.

Figure 10b shows the result of probing a panel of reference nucleic acids comprising the non-region between the H3 and H4 histone genes of various species of fungus,

with a probe generated by amplification of the non-coding region between the H3 and H4 histone genes of a sample containing yeast and human cells.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, cellular biology and recombinant DNA techniques within the skill in the art. Such techniques are well known to the skilled worker, and are explained fully in the literature See, for example, Sambrook and Russell "Molecular Cloning: A Laboratory Manual" (2001); Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridisation" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989). Ausubel, F. et al., 1989-1999, "Current Protocols in Molecular Biology" (Green Publishing, New York).

Before the present methods are described, it is understood that this invention is not limited to the particular materials and methods described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and it is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a nucleic acid" includes a plurality of such nucleic acids, and a reference to "an organism" is a reference to one or more organisms. Unless defined otherwise, all technical and scientific terms used herein

have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

All publications mentioned herein are cited for the purpose of describing and disclosing the protocols and reagents which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

In one preferred embodiment, the invention provides a method for detecting a species of eukaryotic organisms in a sample. As used herein, the term "detecting" means to determine whether one or more species of eukaryotic organism is present in a sample. The method of detection generally involves the amplification of nucleic acid as described herein and subsequent analysis of the amplified product. The analysis may be any method known in the art which permits a comparison between the amplified product and a reference nucleic acid. Suitable analysis methods include, for example, restriction length polymorphism analysis (RFLP) and methods involving hybridisation of nucleic acid sequences such as southern, northern, dot, panel or array hybridisation, sequencing, PCR (including, for example, real-time PCR, nested PCR). By determining whether one or more species are present in the sample, the method of the present invention preferably provides a means for identifying a species of eukaryotic organism in a sample. The term "sample" refers to any material for or from a biological process where a person wishes to detect whether the sample comprises or contains one or more eukarytoic organisms. For example, a sample may be a clinical sample such as a blood, urine or tissue sample from a human, a food sample, a food ingredient sample, a

sample from a wine or beer making process, a sample from a cheese making process, a veterinary sample such as blood, urine or tissue from an animal, an environmental sample such as water or soil, or the sample may be an isolated or purified organism.

The sample may comprise components in addition to eukaryotic organisms such as, for example, fluids, soil, cellular debris, prokaryotic organisms such as bacteria and any other material that may be present in the sample but which is not eukaryotic organisms. Alternatively, the sample may consist of purified or isolated eukaryotic organisms that have been separated or purified from material prior to applying the method of the invention.

Once a sample is obtained, nucleic acid is preferably extracted from the sample. As used herein, "nucleic acid" refers to deoxyribonucleic acid and ribonucleic acid in all their forms, ie. single and double-stranded DNA, mRNA, and the like. The nucleic acid may be extracted using any methods known in the art for extracting nucleic acid from samples containing eukaryotic cells in a form that is suitable for primer based amplification. For example, the nucleic acid may be extracted using methods involving extracting the sample with phenol and/or phenol chloroform, or using nucleic acid binding based technology to extract the nucleic acid from the sample.

Any nucleic acid molecules isolated from the sample which comprise a non-coding sequence located between and contiguous with the coding sequence of the first and second histone genes may be used in the method of the invention. Preferably, the nucleic acid molecule comprises at least 15 base pairs of a coding sequence of a first histone gene of a pair of divergently transcribed histone genes. More preferably, the nucleic acid molecule comprises at least 15 base pairs of a coding sequence of a first and second histone gene of the pair of divergently transcribed histone genes.

As used herein, the term "coding sequence" refers to nucleic acid which encodes a protein or peptide sequence. The term "non-coding sequence" refers to nucleic acid sequence which does not encode a protein or peptide sequence, but may include promoter sequence and intergenic sequences. The expression "divergently transcribed histone genes" refers to two histone genes which lie adjacent each other and in which the direction of transcription of each gene is outward, or in other words diverges, from a central region comprising the promoter for each gene. In most eukaryotic organisms, the most common divergently transcribed histone genes pairs are the H2a/H2b gene pair and the H3/H4 gene pair, however, it will be appreciated by persons skilled in the art that the present invention applies to any divergently transcribed histone gene pairs. It will also be appreciated that eukaryotes that do not possess divergently transcribed gene pairs (such as dinoflagellates and flowering plants such as Arabidopsis) fall outside the scope of this invention.

Following extraction of the nucleic acid from the sample, a first primer is provided. As used herein, the term "primer" refers to a short-length, single stranded polydeoxynucleotide that is chemically synthesised by known methods (involving, for example, triester, phosphoramidite, or phosphonate chemistry), such as described by Engels, et al., Agnew. Chem. Int. Ed. Engl. 28: 716-734 (1989). They are then purified, for example, by polyacrylamide gel electrophoresis. The sequence of the primer may be selected such that the primer is substantially complementary to a target sequence and therefore capable of hybridising to the target. Once the primer is hybridised to the target it may be extended by the addition of deoxyribonucleotides to the 3′ end of the primer using a DNA polymerase, or by the addition of ribonucleotides using an RNA polymerase.

The first primer is complementary to the first strand

of the coding sequence of a first histone gene of the pair of divergently transcribed histone genes. As used herein, "complementary" refers to a nucleic acid sequence that comprises a base sequence that is capable of forming Watson-Crick base pairs with its complementary nucleic acid. The "first strand" is that strand of a double stranded nucleic acid molecule which orients the primer such that the 3′ end of the primer is directed towards the non-coding sequence, and consequently extension of the primer using known polymerases results in extension of the primer into the non-coding sequence. Thus, a primer that is complementary to a first strand will hybridise to the first strand by Watson-Crick base pairing, and extension of the primer will result in synthesis of a strand of nucleic acid that is complementary to a first strand of the non-coding sequence.

Preferably, a first and second primer are provided in the method of the invention. The sequence of the second primer is designed to be complementary to the second strand of the coding sequence of the second histone of the pair of histone genes. The "second strand" is that strand of a double stranded nucleic acid molecule that is complementary to the first strand and which is in the opposite direction to the first strand. As a consequence, the second primer is oriented in the opposite direction to the first primer and consequently extension of the primer using known polymerases results in extension of the primer in the opposite direction to that of the first primer. Thus, extension of the first and second primer results in the synthesis of complementary strands.

The sequence of the second primer may be determined in the same manner as the sequence of the first primer. However, the second primer must be complementary to the second histone gene of a histone gene pair. Thus, if the first primer is complementary to the coding sequence of, for example, the H2a gene, then the second primer must be complementary with the coding sequence of the H2b gene.

Similarly, if the first primer is complementary with the H2b gene, the second primer must be complementary with the H2a gene. If the first primer is complementary with the coding sequence of the H3 gene, then the second primer pair must be complementary with the coding sequence of the H4 gene, and if the first primer is complementary with the coding sequence of the H4 gene then the second primer must be complementary with the coding sequence of the H3 gene.

The primers may be any length provided they can hybridise specifically with a substantially complementary sequence, and do not interfere with the desired specificity of the probe. In a preferred embodiment, the primers are between 10 and 100 nucleotides, more preferably 10 and 40bp in length, even more preferably 15 to 28 base pairs in length.

Preferably, the coding sequence to which the primers are complementary encodes a portion of the amino acid sequence from a histone protein selected from the group comprising H2a, H2b, H3 and H4. Preferably, the primers are complementary to coding sequence which encodes the following portions of amino acid sequence of the indicated histone proteins:

Histone protein	Amino acid sequence (SEQ ID NO)
H2a	VGAGAPVYLTAVLEY (1)
H2a	GAPVYLT (2)
H2a	GNVTIAQGGVLPN (3)
H2a	QGGVVPN (4)
H2a	APVYLTAAV (5)
H2b	VLKQTHPDTG (6)
H2b	QTAVRLILPGELAKH (8)
H2b	QTHPDTG (7)
H2b	PGELAKH (9)
H3	MARTKQTA (10)
H3	PGTVALRE (11)

H3	ALREIRRYQ (12)
H3	GGVKKPHRY (13)
H3	GGKAPRKQ (14)
H4	GITKPAIRR (15)
H4	GKGGKGLGKGG (16)
H4	GKGGAKRHR (17)
H4	MSGGKSGGK (18)
H4	QGITKPAIRR (19)

As used herein, the following single letter codes are used for amino acids:

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic Acid
B	Aspartic acid or Asparagine
C	Cysteine
Q	Glutamine
E	Glutamic Acid
Z	Glutamic Acid or Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine
-	unknown or other

More preferably, the first primer is complementary to coding sequence which encodes the amino acid sequence

selected from the group consisting of SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 5, and the second primer is complementary to coding sequence which encodes the amino acid sequence selected from the group consisting of SEQ ID NO. 7, SEQ ID NO. 9.

The sequence of the first and second primers may be a degenerate sequence to account for wobble in the third codon, or may be an exact sequence match to the coding region. Preferably, the primer sequences are degenerate sequences. As used herein, the term "degenerate" refers to a primer nucleotide sequence in which one or more of the base pairs in the primer sequence may vary between any two or more of the 4 bases A, C, G and T to account for the degeneracy of the genetic code.

For example, to amplify the non-coding sequence of the H2a/H2b histone gene pair, the first primer may comprise a sequence selected from the group:

H2AR GTTAGATANACNGGNGCNCC (SEQ ID NO. 21)
YeastH3ID CTTGCAGTTTGYTTRGTDCKNGCC (SEQ ID NO. 22)
H2R1 GYTARRTAARCKGGAGCACC (SEQ ID NO. 23)
H2AR1 GYTARRTAARCKGGAGCACC (SEQ ID NO. 26)
; and

the second primer may comprise a sequence selected from the group:

H2BR CCAGTGTCNGGRTGNRYYTG (SEQ ID NO. 20)
YeastH4ID CCTTTTCCACCTTTWCCNCTDCCRG (SEQ ID NO. 24)
H2B1 CCAGTATCAGGRTGNACNTG (SEQ ID NO. 25)
H2BRYEAST CCAGTGTCAGGYTGNGTYTG (SEQ ID NO. 27)
H4FIL AATAGCGGGYTTNGTDATNCCYTG (SEQ ID NO. 28).

To amplify the non-coding sequence of the H3/H4 gene pair, the first primer may comprise a sequence selected from the group :

H3P1 GGCNMGNACTAANCAAACAGCTAG (SEQ ID NO. 29)
H2Bfil GCNCTNCGNGARATTCGTCGCTAC (SEQ ID NO. 30)
H3P3 CCNGGNACNGTNGCCTTGAGAG (SEQ ID NO. 31)
H3P4 GARGAYACYAAYYTNKGCGCTATCCAC (SEQ ID NO. 32)
H3P5 GGNGGNGTNAARAAGCCYCAYAG (SEQ ID NO. 33)

H3P6 GGNGGNAARGCNCCRAGAAAGCAACTAGC (SEQ ID NO. 34)
H3R1YEAS CTTTCTTGCTGTYTGYTTNGTYCTNGC (SEQ ID NO. 35)
H2AFIL GCNCCNGTNTAYCTCGCTGCTGTT (SEQ ID NO. 36)
H3R2YEAS CTCTCAAGGCNACNGTNCCNGG (SEQ ID NO. 37)
H3FIL GTAGCGACGAATYTCNCGNAGNGC (SEQ ID NO. 38);

and the second primer may comprise a sequence selected from the group:

H4P1 GGNATNACNAARCCRGCTATCAGTCGG (SEQ ID NO. 39)
H4P2 GGNAARGGNGGNAARGGTCTGGGAAAAGGTGG (SEQ ID NO. 40)
H4P3 GGNAARGGNGGNGCYAAACGTCATAG (SEQ ID NO. 41)
H4P4 GAYAAYATHCARGGGATCACGAAG (SEQ ID NO. 42)
H4P5 CARGGNATHACRAARCCRGCTATCCGACG (SEQ ID NO. 43)
H4R1YEAS GATAGCTGGYTTNGTNATNCC (SEQ ID NO. 44)
H4R1FIL GATAGCTGGYTTNGTNATNCC (SEQ ID NO. 45).

The first and second primer may also be non-degenerate primers. For example, to amplify the non-coding region between H2A and H2b, the first primer may be:

H2Acere GTTAGATAGACTGGAGCACC (SEQ ID NO. 46);

and the second primer:

H2BRCere CCTGTGTCTGGGTGAGTTTG (SEQ ID NO. 47).

To amplify the non-coding region between H3 and H4, the first primer may be:

H3R1CERE CTTTCTTGCTGTTTGCTTTGTTCTGGC (SEQ ID NO. 48);

or

H3R2CERE CTCTCAAGGCAACAGTACCTGG (SEQ ID NO. 49)

and the second primer:

H4R1CERE GATAGCTGGCTTAGTGATACC (SEQ ID NO. 50).

As used herein, the following standard abbreviations for nucleotides are used:

a	a	adenine
g	g	guanine
c	c	cytosine
t	t	thymine
u	u	uracil

r	g or a
y	t/u or c
m	a or c
k	g or t/u
s	g or c
w	a or t/u
h	a or c or t/u
b	g or t/u or c
d	g or a or t/u
n	a or g or c or t

Non-degenerate primers that are complementary to the degenerate primers or the non-coding sequence may also be used to either amplify the non-coding region, or further amplify the nucleic acid sequence that has been amplified using the degenerate first and second primers to amplify non-coding sequence from specific organisms. Examples of non-degenerate primers complementary to the coding sequence which are capable of amplifying the non-coding sequence of specific organisms are as follows:

Coding region	Organism	Primer Sequence	SEQ ID NO.
H2a	C. utilis	GGGAAAGTCAAACCTGCCTTAGCAGAAC	51
	P. membraneafaciens	GGGAAAGTTAAACCAGCCTTAGAAGATC	52
	K. marxianus	GGGAAGGTCAAACCTGCCTTGGCAGATC	53
	S. cerevisiae	GGGAATGTTAAACCAGCTTTAGCAGATC	54
	C. lusitaniae	GGGAAAGTCAAACCTGCCTTAGCAGATC	55
	K. marxianus	GGGAAAGTCAAACCTGCCTTAGCAGATC	56
	Z. rouxi	GGGAAGGTCAAACCTGCCTTAGCAGATC	57
	H. uvarum	GGGAAGACTAAGTTAGCCTTAGCAGATC	58
	D. hansenii	GGGAAAGTTAACCCAGCCTTAGCAGATC	59
	S. cerevisiae	GGGAATGTCAAACCAGCCTTAGCAGATC	60
	T. delbrueckii	GGGAAAGTTAGACCAGCCTTAGCAGATC	61
	D. anomala	GGGAAAGTTAATCCAGCTCTTGCGGATC	62

	P. guillermondii	GGGAATGTCAATCCAGCCTTAGCGAATC	63
	C. multigemmis	GGGAAAATTAAACCAGCTTTTGATGATC	64
	P. guillermondii	GGGAAAGTCAAACCAGCCTTAGCCGATC	65
	C. tropicalis	GGGAAAGTCAAACCAGCTTTAGCTGATC	66
	T. delbrueckii	GGGAAAGTTAGACCAGCCTTAGCTGATC	67
	C. parapsilosis	GGGAAAGTTAATCCAGCCTTGGCTGATC	68
	E. fibuliger	GGGAAAGTCAAACCAGCTTTGGCTGATC	69
	Z. bisporus	GGGAAGGTTAAACCAGCCTTGGCTGATC	70
H2b	Primer 1		
	C. lusitaniae	CCTGTGTCTGGGTGAGTTTGCTTCAACAC	71
	C. multigemmis	CCTGTGTCTGGGTGAGTTTGTTTTAAAAC	72
	C. parapsilosis	CCTGTGTCTGGGTGAGTTTGTTTCAAAAC	73
	C. tropicalis	CCTGTGTCTGGGTGAGTTTGTTTCAAGAC	74
	C. utilis	CCTGTGTCTGGGTGAGTTTGCTTCAAAAC	75
	D. anomala	GTCAGGTTGGGTTTGCTTCAACAC	76
	D. hansenii	CCTGTGTCTGGGTGAGTTTGTTTTAAAAC	77
	E. fibuliger	CCTGTGTCTGGGTGAGTTTGTTTTAAAAC	78
	Hanseniaspora uvarum	CCTGTGTCTGGGTGAGTTTGCTTTAAGAC	79
	K. marxianus	CCTGTGTCTGGGTGAGTTTGCTTCAAGAC	80
	K. marxianus	AGTGTCAGGCTGGGTTTGCTTCAAAAC	81
	P. guillermondii	CCTGTGTCTGGGTGAGTTTGCTTCAACAC	82
	P. guillermondii	TGTGTCTGGGTGAGTTTGCTTCAAGAC	83
	P.membraneaf aciens	CCTGTGTCTGGGTGAGTTTGTTTTAAAAC	84
	S. cerevisiae	CCTGTGTCTGGGTGAGTTTGCTTCAAAAC	85
	S. cerevisiae	CCTGTGTCTGGGTGAGTTTGCTTCAAAAC	86
	T. delbrueckii	CCTGTGTCTGGGTGAGTTTGTTTCAAAAC	87
	T. delbruekii	CCTGTGTCTGGGTGAGTTTGTTTCAAAAC	88
	Zygosaccharomyce s bisporus	CCAGTGTCAGGTTGAGTCTGCTTCAAAAC	89
	Z. rouxii	CCTGTGTCTGGGTGAGTTTGCTTCAAAAC	90

	Primer 2	Primer 2	
	C. lusitaniae	CCTTGTAGATGTAAGAAGAGTAAGTCTC	91
	C. parapsilosis	CTTTGTAAATATATGATGAATAAGTCTC	92
	C. utilis	CCTTGTAGATGTAAGAGGCGTAAGTCTC	93
	K. marxianus	CCTTGTAGATGTAAGAGGAGTAAGTCTC	94
	P. guillermondii	CCTTGTAGATGTAAGAAGAGTAAGTCTC	95
	D. anomala	CCTTGTAAATGTATGAAGACCAACTCTC	96
	S. cerevisiae	CTTTGTAGATATAAGAGGAATAGGTCTC	97
	Z. bisporus	CTTTGTAAATATAAGAAGAGTAGGTCTC	98
	Z. rouxii	CCTTGTAAATGTAAGAGGAGTTGGTCTC	99
	C. multigemmis	CTTTGTAAATATAAGAAGAATAAGTTTC	100
	C. tropicalis	CTTTGTAAATGTATGAGGAGTAAGTTTC	101
	D. hansenii	CCTTGTAAATGTAAGAAGAGTAAGTTTC	102
	E. fibuliger	CTTTATAGATATAAGAAGAGTAAGTTTC	103
	K. marxianus	CCTTGTAGATGTAAGAAGAGTAAGTTTC	104
	P. membraneaf aciens	CTTTATAAATATATGATGAATAAGTTTC	105
	T. delbrueckii	CTTTGTAGATGTAAGAAGAATAAGTTTC	106
	BP	CTTTGTAAATGTAAGAGGAGTAAGTTTC	107
	T. delbrueckii	CCTTGTATATGTACGAAGAGTATGTTTC	108
	CS	CTTTGTAAATGTAAGAAGAGTATGTTTC	109
	P. guillermondii	CCTTGTAGATGTAGATACCGAAGGTTTC	110
	S. cerevisiae		
	Hanseniaspora uvarum		
H3	C. multigemmis	GTTTTCTTGGAGCTTTACCACCAGTGG	111
	C. tropicalis	GTTTTCTTGGTGCTTTACCACCGGTAG	112
	C. utilis	GCTTTCTTGGGGCCTTACCACCAGTGG	113
	C. lusitaniae	GCTTTCTTGGGGCCTTGCCACCAGTGG	114
	D. hansenii	GTTTTCTTGGGGCTTTACCACCAGTGG	115
	H. uvarum	GTTTTCTTGGAGCCTTACCACCGGTGG	116
	K. marxianus	GTTTTCTTGGTGCTTTACCACCAGTGG	

	K. marxianus	GCTTTCTTGGGGCCTTGCCACCGGTGG	117
	M. pulcherrima	GCTTTCTTGGGGCCTTACCACCAGTAG	118
	P. anomala	GTTTTCTTGGTGCTTTACCACCAGTGG	119
	P. guillermondii	GCTTTCTTGGGGCCTTACCACCAGTAG	120
	P.	GTTTTCTTGGAGCTTTACCACCAGTTG	121
	membraneafaciens		122
	S. pombe	GCTTACGAGGAGCCTTACCACCAGTTG	123
	R. mucila	GCTTGCGGGGAGCCTTTCCTCCGGTCG	124
	R.rubra	GCTTGCGGGGAGCCTTTCCTCCGGTCG	125
	S. cerevisiae	GTTTTCTTGGGGCTTTACCACCAGTAG	126
	T. delbrueckii	GCTTTCTTGGTGCTTTACCACCAGTGG	127
	CS	GCTTTCTTGGTGCTTTACCACCAGTGG	128
	T. delbrueckii	GCTTTCTTGGGGCCTTACCACCAGTAG	129
	BP	GCTTTCTTGGAGCCTTACCACCAGTAG	130
	Z. rouxii		
	Z.bailli		
H4	K marxianus	CCTTTTCCTCCTTTTCCTCTGCCAGACCCTT	131
	M. pulcherrima	TTCCTCCTTTTCCTCTGCCAGAC	132
	P. guillermondii	CCTTTTCCGCCTTTTCCTCTTCCAGAC	133
	C. lusitaniae	CCTTTTCCACCTTTTCCTCTGCCGGAC	134
	R. mucilaginosa	CCCTTGCCACCCTTTCCACGGCCAGAC	135
	R. rubra	CCCTTGCCACCCTTTCCACGGCCAGAC	136
	P.membraneafaciens	CCTTTTCCACCTTTTCCTCTACCAGAC	137
	T.delbrueckii BP large	CCTTTACCACCTTTTCCTCTACCAGAC	138
	T. delbrueckii BP small	CCTTTACCACCTTTTCCTCTACCAGAC	139
	Z. bisporus	CCTTTACCACCTTTTCCTCTACCAGAC	140
	C. multigemmis	CCTTTTCCTCCTTTTCCTCTACCAGAC	141
	Z. bailli	CCTTTACCACCTTTACCTCTACCAGAC	142
	D. hansenii	CCTTTTCCACCTTTTCCTCTACCGGAC	143

	P. anomala	CCTTTACCACCTTTTCCTCTACCGGAC	144
	Z. rouxii CS	CCTTTACCACCTTTTCCTCTACCGGAC	145
	Z. rouxii BP	CCTTTACCACCTTTTCCTCTACCGGAC	146
	C. utilis	CCTTTACCACCTTTACCTCTACCGGAC	147
	Hanseniaspora uvarum	CCTTTACCACCTTTACCTCTACCGGAC	148
	K. marxianus BP	CCTTTACCACCTTTACCTCTACCGGAC	149
	S.cerevisiae	CCTTTACCACCTTTACCTCTACCGGAC	150
	T.delbrueckii CS	CCTTTACCACCTTTACCTCTACCGGAC	151
	S. pombe	CCTTTTCCACCTTTACCACGGCCAGAC	152

Once the sequence of the primer(s) is designed, the primers are contacted with the nucleic acid. As used herein, the term "contacted" means incubating the primers with the nucleic acid in conditions to permit hybridisation or annealing of complementary sequences. The conditions which permit annealing of complementary sequences will depend on the sequence and length of the primer. For example, the temperature of annealing is affected by the GC content of the primer. Preferably, the primers are incubated with the nucleic acid at a temperature between 30°C and 72°C to permit annealing of the primer(s). More preferably, the primers are annealed at a temperature of between 45°C and 72°C. Most preferably, the primers are annealed at a temperature of between 45°C and 65°C.

Once the primer(s) are contacted with the nucleic acid, at least a segment of the non-coding sequence is amplified. As used herein, the term "amplified" refers to the synthesis of at least one nucleic acid strand that is complementary to a template nucleic acid strand by extension of a primer from the 3′ end of the primer. A segment of the non-coding sequence may be amplified by any methods know to those skilled in the art. Preferably, the first and second primer is used in a PCR amplification to

amplify the non-coding sequence. A person skilled in the art will recognize, however, that amplification of non-coding sequences in a sample may be accomplished by any known method using a first primer or a first and second primer where appropriate techniques such as primer extension, ligase chain reaction (LCR), QP-replicase amplification, transcription amplification, and self-sustained sequence replication, each of which provides sufficient amplification. "Polymerase chain reaction," or "PCR," as used herein generally refers to a method for amplification of a desired nucleotide sequence *in vitro*, as described in U.S. Patent No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using a first and second primer capable of hybridizing preferentially to a target nucleic acid. Typically, the primers used in the PCR method will be complementary to nucleotide sequences within the template at both ends of or flanking the nucleotide sequence to be amplified, although primers complementary to the nucleotide sequence to be amplified also may be used. Wang, et al., in PCR Protocols, pp.70-75 (Academic Press, 1990); Ochman, *et al.*, in PCR Protocols, pp. 219-227; Triglia, *et al.*, *Nucl. Acids Res*. 16:8186 (1988).

The reaction conditions for the extension reaction such as annealing time and temperature and extension time and temperature will vary depending on the sequence of the primer and the nature of the polymerase used in the extension reaction. The appropriate reaction conditions to be used may be determined as described in Wang, et al., in PCR Protocols, pp.70-75 (Academic Press, 1990); Ochman, *et al.*, in PCR Protocols, pp. 219-227.

It is also contemplated that the non-coding region may be amplified using real-time PCR assays using reference primers which are labeled with interactive fluorescent label pairs such as two fluorophores or a fluorophore and a non-fluorescent quencher, such that a change in fluorescence signal indicates the presence of reference

primers that have been extended and, thus, the presence of a target for the reference primers in a sample. The term "real-time analysis" refers to periodic monitoring during PCR. Certain systems such as the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif) conduct monitoring during each thermal cycle at a pre-determined or user-defined point. Interaction between labels may be by fluorescence resonance energy transfer (FRET), by touching, or both. The changes in flourescence may be utilised to quantitate the number of eukaryotic organisms in a sample. The fluorecent labelled primers may also function to label the probe for detection following hybridisation. Fluorescence-based approaches to provide end-point or real-time measurements of PCR amplification products (amplicons) (Holland etal, (1991) Proc. Natl. Acad. Sci., 88:7276-80) may employ intercalating dyes, e.g. ethidium bromide, to indicate the amount of double stranded DNA present (Gelfand etal, U.S. Pat. No. 5,210,015) (for example, SYBR Green I from Molecular Probes) or probes containing reporter-quencher pairs ("TaqMan.RTM.", 5' nuclease assay) that are cleaved during amplification to release a fluorescent signal proportional to the amount of double stranded DNA present (Livak etal, U.S. Pat. No. 5,538,848; Gelfand etal, U.S. Pat. No. 5,804,375). Fluorescent dyes useful for labelling primers and nucleotide 5'-triphosphates may include, for example, fluoresceins, rhodamines (e.g., U.S. Pat. Nos. 5,366,860; 5,936,087; 6,051,719), cyanines (U.S. Pat. No. 6,080,868 and WO 97/45539), and metal porphyrin complexes (WO 88/04777). Examples of fluorescein dyes include 6-carboxyfluorescein (6-FAM) 1, 2',4',1,4,-tetrachlorofluorescein (TET) 2 and 2',4',5',7',1,4-hexachlorofluorescein (HEX) 3 (U.S. Pat. No. 5,654,442), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyrhodamine (JOE) 4, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein 5 (U.S. Pat. Nos. 5,188,934 and

5,885,778), 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein 6 (U.S. Pat. No. 6,008,379).

Quenchers may include, for example, rhodamine fluorescent dyes selected from the group consisting of tetramethyl-6-carboxyrhodamine (TAMRA) 7, tetrapropano-6-carboxyrhodamine (ROX) 8, diazo compounds, e.g. 9-11, and cyanine dyes including 11, anthraquinone, malachite green, nitrothiazole,and nitroimidazole compounds and the like.

Following amplification, a probe results that is complementary to one or more non-coding sequences present in the sample. As used herein, the term "probe" refers to a nucleic acid molecule having a nucleotide sequence that is substantially complementary to its target nucleic acid sequence to be able to form a detectable hybrid probe:target duplex under high stringency hybridisation conditions. A "probe:target' duplex" is a structure that is a double-stranded structure formed between two complementary nucleic acid molecules. The structure is sufficiently stable to withstand wash conditions following hybridisation and to be detected by means of radioisotopes, chemiluminescent molecules, enzymes that may be bound to the probe such as digoxigenin, luciferase, alkaline phosphatase or haptens.

The probe may be of any length that is sufficient to permit the probe to hybridise specifically to a reference nucleic acid of the same species from which the probe is derived. Preferably, the probe comprises at least 15 base pairs of non-coding sequence. More preferably, the probe comprises at least 50 base pairs of non-coding sequence. Even more preferably, the probe comprises at least 300 base pairs of non-coding sequence.

The probe may be compared with one or more reference nucleic acid molecules using any method which permits comparison of the sequence of the probe with the sequence of one or more reference nucleic acid molecules to allow detection of a eukarotic species. For example, the probe may be compared with the one or reference nucleic acids by

restriction length polymorphism analysis of the probe and reference nucleic acid or by hybridisation of the probe to reference nucleic acid.

Preferably, the probe is hybridised with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species. The reference nucleic acid molecule may be any sequence that is complementary to the non-coding sequence of a pair of divergently transcribed histone genes of a known species. Thus, hybridisation of the probe to the reference nucleic acid under high stringency conditions is indicative that the sample comprises an organism of that species.

As used herein, the expression "reference nucleic acid" refers to any nucleic acid that contains nucleic acid that is complementary to the non-coding region of a pair divergently transcribed histone genes. The reference nucleic acid may be, for example, genomic DNA isolated from any eukaryotic organism having a pair of divergently transcribed histone genes. The isolation method is not critical to the invention provided the nucleic acid is in a form that is capable of hybridising to substantially complementary sequence. The reference nucleic acid may be nucleic acid that has been previously isolated or amplified from the non-coding sequence between the coding sequence of a pair of divergently transcribed histone genes using the method of the invention or any other method. The reference nucleic acid may be reference primers that are complementary to the sequence of the non-coding region.

In one embodiment, one or more first reference primers may be used which are capable of hybridising to a non-coding region of a particular species. As used herein, a "reference primer" is a primer that is complementary to the non-coding sequence located between and contiguous with a pair of divergently transcribed histone genes. By contacting the reference primer with

the amplified probe under condition for extension of the reference primer, hybridisation of the reference primer to the amplified probe can be detected by detecting extending the hybridised primer and detecting the extension product. An extension product is only produced if the primer hybridises to the amplified probe and therefore detection of an extension product generally indicates hybridisation of the reference primer. The reference primer may be extended in any manner that adds nucleotides to the primer in a template dependent manner. Preferably, a first and second reference primer is used in a PCR reaction. Extension of the primer may be detected by any method useful for detecting addition of nucleotides to the primer. For example, extension of the primer may be detected by any of the following methods: visualisation of the extended primer by gel electrophoresis and staining, flourophore labelling of the nucleotides or the primer such as fluorescent labelling, real-time PCR, incorporation of one or more radioactive nucleotides, or any other method known in the art for detection of extended primers. Examples of first and second reference primers are as follows:

Histone Gene pair	Organism	First and second reference Primer Sequence	Length of amplified fragment	SEQ ID NO
H2a/H 2b	C. lusitaniae	GAATGCAGGAGAATATCCGCGTCGC TCTCCAATCAGTGAGTGCGTTTTGCC	218 bp	153 154
	C. multigemmis	GTATCCCTTTACCTGAGAGTTGTCTCG TCGTGACTTGTCATCAAAGTGGCGTC	334 pb	155 156
	C. parapsilosis	CTTCTCTTCTTCTTCTCCTCCGACTC GAAACAGTGAGATTGGGAGAGTGGCG	280 bp	157 158
	C. tropicalis	GGTTAGGATGGCGGATTCACAATC GTTTGCCACGGATTTTCTTTGTGC	420 bp	159 160

C. utilis	CAATTCCCACGAGTGAACGCGTTTAC GAATGTTGGGACTTGGCAGAAGTGTG	252 bp	161 162
D. anomala	GAACGAAAACAAGGGCAAAGGGAAGC GGAAATGACCAGCATGTCATCAACCG	324 bp	163 164
D. hansenii	AAACTACCCACAGGGAATACAATCGCC GTTTACTTAACAGCCATCTGATACGGG	246 bp	165 166
E. fibuliga	TTTAACTCTGCGTGATGTAGCGGTGC GAATCGTCCCAGTTATAAACCCGCCAG	221 bp	167 168
Hanseniaspora uvarum	CGATCATACGTGTATGCTTAGGAACC GCGTTTCTTGTGGTTTTTCTCTATCC	319 bp	169 170
K. marxianus	GAGAAAACCCCACTTACAATATGCG CAGGAGGGTACAACTAGCTACCACTC	380 bp	171 172
K. marxianus	CGTTAAGTATGGCGAGACGAGTCGTG CAAAAGCAACGTGACGCCACTGTACC	367 bp	173 174
P.guillermondii	GAATCAACCAAAACATGGAGTCTGCGG AAGAAGTCCCGTCACGAGCTATCGTC	327 bp	175 176
P.guillermondii	ACCGCATCTTTATGAATGCTCCCTTC ATACAATGCGCAAATCGTACAGATCG	219 bp	177 178
P. membraneafaciens	AAGGAGGGTGGCTCATCTATCAAGAC TAACATGAATAGAGGGGAAGTACAGC	267 bp	179 180
S. cerevisiae	GACGGCAAGTGTCTCACTGTTGCATTACG CGAAAATGGTAGCACGTCGCGTTTATGG	247 bp	181 182
S. cerevisiae	CATCAAGAGAACAGAGATGCCCCTTTC GTATTTACGATCCACTGGCTGGCTTCG	479 bp	183 184
T. delbruekii	CTCTCAATACCTCCAATAAGGCCTCC CCTTCACCGTGTGCTGTGAAAAACC	302 bp	185 186
T. delbruekiiCS	GAAAGGACAAGAAACACCGGGATCGAC CACGATTACTAGCTCTTCGACTGTCC	271 bp	187 188
Z. bisporus	CTGCGAATGTTCAGCGTAAGTTTCAGC CAAGAGTCTCCGACTACAGTTACACC	253 bp	189 190

	Z. rouxii	GGATCACCGGGTGGGTAGTTGTAAGG CTCACCCGGTGTGAAATAACTGGAACG	271 bp	191 192
H3/H4	C. lusitaniae	ATAGAGGGTGGTGGAATGTGATGCTC GGAAAAACAAGCGCGCGAAGAAATAC	337 bp	193 194
	C. Utilis	CACCACCACACGTCTTCAACACTCGC GTTACACAGGTGTTTACCCTAGTAGG	301 bp	195 196
	D. hansenii	CCATGTCATCCTATTCTATCCCGTG CTGTGCGAGGTAACTATCAATAGGCC	309 bp	197 198
	Hanseniaspora uvarum	CCTGTGGATTGTGTTCTGTACATTG GTGTCTAGTCAAAGTGTGTTTTGCC	223 bp	199 200
	K.marxianus	GTCGTGTTTGCAGGTGGTTTCTGTTG CTGACCGCAAATGGCCATAGTATTCG	325 bp	201 202
	K.marxianus	GTGGAGAAACAAAAGCGAGCCTTTGC GTCAGCTGTTGCCATAATTCACGTTCC	299 bp	203 204
	M. pulcherrima	CTTAAGTTTCGAGACCGAGAATGACGC AGAGGCCACAGATGCATTCTTTTGTG	311 bp	205 206
	P. anomala	GATCCAACTTGTTGATATCGCGCCTG CAAAGATGGACATGGTGGCGTGCTGAG	264 bp	207 208
	P.guillermondii	GCGCACCGGCTGCCTCTTTATATAGAG CTTGCAGTATGTCACGGTACCAATATC	307 bp	209 210
	P. membranaefaciens	CGAGTGATATAAATTGGGGGGATATGGTG GTGTAAAATTGATACCAGACCCTCAGTCTC	272 bp	211 212
	R. mucilaginosa	GAGAGTCAGTTAGAGTCGTGTGCGAG GTGTCGTAGCTCTTGCACGAACCTAGG	315 bp	213 214
	S. cerevisiae	CGCACAAACACGTATGTATCTAGCCG CTTAATGCGAAGTGCTCGGAACGGAC	340 bp	215 216
	C. albicans	GTTTGTGCCTGGTGCGAAATGG GGGAGTAAAGGAAGAAGAGTTC	220 bp	217 218
	T.delbruekii CS	CTAGGATATCCCTCAAATTCGCCGCTG GATCACATTTTTGCGGTCTCTCCACC	308 bp	219 220

Z. rouxii	CAGCTGTGAGAAGGAGTTATTGACTC CGTTACTGGTATACGAGCATGCTCAG	214 bp	221 222
R. rubra	GAGAAGAGTGAGGTAAGAGAGCGAAG GAAAGGAGAGAGAGCAAGCGTTGTTTG	320 bp	223 224
C. multigemmis	GAAGGTACAGGAGACATATATCGCGAC CGTGAATAGGTTTATTGTGGTGTGCG	290 bp	225 226
S. pombe	GCTAGTAGGGTATGCGGCATTTCAC GTGTTCTGATAATTGCCCTGTGTAG	173 bp	227 228
Z. bisporus	AGGTTTCAACAGCCAATGGTGATGCG GAAAGGAGTAGACTACAGGGAGGTTC	220 bp	229 230
Z. bailii	GAAACGCTGGGAACGTTTTCATTACC GAGAAATTACTCTGGAACGACGCCAC	220 bp	231 232
C. glabrata	GTAATTTGCCACAACCACCAAGCACC GTTGGACGGTATGCGTATTTCAGGCG	307 bp	233 234

It will be appreciated by those skilled in the art that once a probe for a particular species is generated using the method of the invention, sequence of the probe may be obtained using methods known in the art, and subsequently reference primers may be designed that are complementary to the non-coding region of that species.

It is also envisaged that hybridisation of the first and/or second reference primers, including for example those referred to above, could be detected without extending the first and/or second reference primer. This may be achieved, for example, by labelling the primer prior to hybridisation with the amplified probe. Methods for labelling and detection of labelled primers to nucleic acid are well known in the art and are described in, for example, Sambrook and Russell "Molecular Cloning: A Laboratory Manual" (2001); Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985).

The reference nucleic acid may include sequences generated using the first and second primers or functional fragments thereof. As used herein, the expression "functional fragments" refers to fragments that contain sufficient contiguous sequence to permit a person skilled in the art to determine that an amplified sequence is substantially complementary to the fragment. Preferably, a functional fragment is at least 15 base pairs in length. The reference nucleic acid may also be any oligonucleotides that have the sequence of a portion of the non-coding sequence between the coding sequence of a pair of divergently transcribed histone genes.

In another embodiment, the amplified probe is hybridised with a panel of reference nucleic acids. As used herein, a "panel" of reference nucleic acids refers to one or more nucleic acid molecules which are arranged or organised in a manner that is suitable for use in hybridisation. For example, the panel of reference nucleic acid may comprise nucleic acid that is arranged as nucleic acid immobilised in an array, hybridisation membrane, glass slide or microchip. As used herein, "microchip" refers to a support whereby the reference nucleic acid is immobilised on a silicone or glass chip. Techniques borrowed from the microelectronics industry are particularly suitable to these ends. For example, micromachining and photolithographic procedures are capable of producing multiple parallel microscopic scale components on a single chip substrate. Materials can be mass produced and reproducibility is exceptional. The microscopic sizes minimise material requirements. Thus, human manipulations can be minimised by designing a microchip type surface capable of immobilising a plurality of reference nucleic acids on the one microchip surface.

The panel may contain reference nucleic acid from any species of eukaryotic organism which possesses a pair of divergently transcribed histone genes. As used herein,

the term "panel" refers to a plurality of reference nucleic acids organised in a manner whereby the position of each reference nucleic acid is known and the identity of a eukaryotic organism may be determined by matching the position of detectable hybridisation with the known position of a reference nucleic acid.

In one embodiment, the panel may have applications in the identification of, for example, yeast and mycelial fungal species and may therefore include, for example, reference nucleic acid from *Brettanomyces clausenii, Brettanomyces custerii, Brettanomyces anomalous, Brettanomyces naardenensis, Candida himilis, Candida intermedia, Candida saki, Candida solani, , Candida tropicalus, Candida versatilis, Candida bechii, Candida famata, Candida lipolytica, Candida stellata, Candida vini, Debaromyces hansenii, Dekkera intermedia , Dekkera bruxellensis, Geotrichium sandidum, Hansenula fabiani, Hanseniaspora uvarum, , Hansenula anomala, Hanseniaspora guillermondii, Hanseniaspora vinae, Kluyveromyces lactis, Kloeckera apiculata, Kluyveromyces marxianus, Kluyveromyces fragilis, Metschnikowia pulcherrima, Pichia guilliermodii, Pichia orientalis, Pichia fermentans, Pichia membraneafaciens, Rhodotorula, Saccharomyces bayanus, Saccharomyces cerevisiae, Saccharomyces dairiensis, Saccharomyces exigus, Saccharomyces uinsporus, Saccharomyces uvarum, Saccharomyces oleaginosus, Saccharomyces boulardii, Saccharomycodies ludwigii, Schizosaccharomyces pombe, Torulaspora delbrueckii, Torulopsis stellata, Zygoaccharomyces bailli* and *Zygosaccharomyces rouxii, Alternaria alternata, Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus versicolor, Blastomyces dermatiditis, Candida albicans, Candida dubliniensis, Candida krusei, Candida parapsilosis, Candida tropicalis, Candida glabrata, Coccidioides immitis, Cryptococcus neoformans, Epidermophyton floccosum, Histoplasma capsulatum, Malassezia furfur,*

Microsporum canis, Mucor spp., Paracoccidioides brasiliensis, Penicillium marneffei, Pityrosporum ovale, Pneumocystis carinii, Sporothrix schenkii, Trichophyton rubrum, Trichophyton interdigitale, Trichosporon beigelii and *Rhodotorula spp.*.

In another embodiment, the panel may have applications in the identification of, for example, protozoans, algae, cnidarians, annelids, nematodes, ciliates, yeast and mycelial fungi having divergently transcribed histone gene pairs. An example of a protozoan which may be identified includes *Trichomonas* sp..

It is also envisaged that the panel may comprise nucleic acid from organisms which have not been characterised but which have been previously isolated.

The probe will comprise a nucleic acid sequence that is substantially complementary to the non-coding sequence located between the coding sequence of a pair of divergently transcribed histone genes of a species of organism in a sample from which the probe is derived. Thus, the probe is used to identify the species from which it is derived by hybridisation to the panel of reference nucleic acids. The term "hybridization" refers to a well known method whereby under sufficiently stringent hybridization conditions, the probe hybridizes specifically only to substantially complementary sequences. As used herein, a nucleic acid sequence is "substantially complementary" to another nucleic acid sequence if greater than 85% of the sequence is capable of forming Watson-Crick base pairing with the other sequence, preferably 90% of the sequence, more preferably 95% of the sequence and even more preferably 100% of the sequence. A substantially complementary sequence may contain mismatches in the sequence, or may comprise ends such as primer ends which are outside the sequence between the translation start sites, or ends which are added to assist in, for example, cloning of the probe or detection of hybridisation of the probe. Sequences that are

substantially complementary will hybridise under stringent conditions as defined for a particular system. Defining appropriate hybridization conditions is within the skill of the art. See eg. Sambrook et al., DNA Cloning, vols. I, II and III. Nucleic Acid Hybridization. However, ordinarily, "stringent conditions" for hybridization or annealing of nucleic acid molecules are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750mM NaCl, 75mM sodium citrate at 42°C.

Another example is use of 50% formamide, 5 X SSC (0.75M NaCl, 0.075M sodium citrate), 50mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 X Denhardt's solution, sonicated salmon sperm DNA (50µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 X SSC and 0.1% SDS.

Following washing of the hybridisation complexes, the hybridisation complexes are detected according to well known techniques and is not a critical aspect of the present invention. Nucleic acid probes capable of specifically hybridizing to a target can be labelled by any one of several methods typically used to detect the presence of hybridized nucleic acids. One common method of detection is the use of autoradiography using probes labeled with 3H, 125I, 35S, 14C, or 32P, or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability, and half lives of the selected isotopes. Other labels include compounds (e.g., biotin and digoxigenin), which bind to antiligands or antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as

fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The method of the present invention also provides nucleic acid molecules produced by the method of the invention. In one embodiment, the nucleic acid molecules are the probe. As discussed above, the amplified probe will contain a portion of the coding region of at least one of the histone genes. In another embodiment, the nucleic acid molecules are produce by amplification of the non-coding region of the amplified probe using a first, and preferably a second, reference primer that is complementary to the non-coding region. Amplification of the amplified probe using a first and second reference primer results in production of nucleic acid molecules that do not contain coding sequence.

It is envisaged that the first, and preferably the second, reference primers may be used directly on nucleic acid extracted from the sample to identify whether the sample is a particular eukaryotic species. This may be achieved by hybridising the reference primer with nucleic acid from the sample under stringent conditions to permit substantially homologous sequences to hybridise, and extending the reference primer using techniques for extension of a primer as discussed above. This approach results in nucleic acid molecules that can also be used subsequently as probes for detecting the eukaryotic organism from which the non-coding region is derived.

Probes produced by the above methods may be used directly on a sample to determine whether that sample belongs to a particular eukaryotic species. Using this approach, the nucleic acid molecules extracted from the sample are hybridised with probe or probes under stringent conditions which permit hybridisation of substantially homologous sequences. Hybridisation of the probes to the sample nucleic acid may be detected in any manner known in

the art and as discussed above.

Determination of the sequence of the non-coding region can be readily determined using the method of the invention using conventional sequencing methodology well known in the art (see for example, Sambrook and Russell "Molecular Cloning: A Laboratory Manual" (2001); Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985). It will be clear to those skilled in the art that once the sequence of the non-coding region has been determined for each Eukaryotic species, primers can readily be designed that are complementary to the non-coding region of a particular eukaryotic species for use in detection of that eukaryotic species, be it by amplification from an amplified probe, from the nucleic acid extract from the sample, by detection of hybridisation of the primer to the nucleic acid of the sample or by any other means using the primer.

It is also contemplated that the probes produced by the method of the invention may be used in conjunction with known probes for identifying eukaryotic organisms. For example, the probes may be used in conjunction with probes produced from the 16S-23S ribosomal RNA intergenic region.

Also contemplated for use with the method of the invention are kits. As is used herein, the term "kit" refers to a group of components that are capable of being used together to identify one or more species of eukaryotic organism. A kit may include, for example one or more primers or nucleic acid molecules or probes as described above, either in solution or in a dried down form ready for resuspension. The kit may also include reference nucleic acid solution, lyophilised or immobilised on an appropriate substrate such as a hybridisation membrane, glass slide or micro-chip. The kit may also include instructions for use of the kit. The kit may further comprise solutions, buffers and enzymes for amplification of the nucleic acid using the primers,

and/or appropriate solutions, buffers and enzymes for labelling the nucleic acid, and/or buffers, solutions and enzymes for hybridising the amplified nucleic acid to the reference nucleic acid.

The method of the invention may be used to detect eukaryotic species having a pair of divergently transcribed histone genes in any sample. In one embodiment, the method may be used to detect eukaryotic organisms in food samples such as yoghurt, brewed products, dairy products, meat products, bakers products etc.

The method of the invention may be used to detect eukaryotic species having a pair of divergently transcribed histone genes in any plant samples. Thus, the method of the invention may be used for the diagnosis of diseases in plants associated with eukaryotic species having a pair of divergently transcribed histone genes. Use of the method in the diagnosis of plant diseases associated with eukaryotic organisms having divergently transcribed histone genes has the added advantage that higher plants do not have divergently arranged histone genes. Examples of plant diseases which may be diagnosed using the method of the invention include fruit rot (caused by, for example, *Penicillium expansum, Monilinia fruitigena*), anthracnose (caused by *Colletotrichum musae*), disease caused by *Botrytis cineria*, potato blight (caused by *Phytophthora infestans*), dutch elm disease (caused by *Ophiostoma ulmi*), Panama disease (caused by *Fusarium oxysporum*), rust (caused by, for example, *Puccinia graminis, Puccinia striiformis, Puccinia recondita, Phradmidium violaceum, Rubus fruticosus, Puccinia punctiformis*), powdery mildew (caused by, for example, *Sphaerotheca pannosa, Podosphaera oxyacanthae, Erysiphe graminis*).

The method of the invention may be used for the diagnosis of disease in subjects, wherein the disease is associated with a eukaryotic species having a pair of

divergently transcribed histone genes. For example, the non-coding region between the H2a-H2b and/or H3-H4 histone gene pairs may be amplified from a sample, and the resulting probe used to hybridise to a panel of nucleic acids which comprise the non-coding region between the H2a-H2b and/or H3-H4 histone gene pairs of known organisms to thereby identify the eukaryotic species present in the sample. The subject may be human, or any animal.

As discussed above, the inventors have further found that by using primers complementary to the coding region of the H3 and H4 histone genes, the non-coding region corresponding to that of the humans is not amplified. Without wishing to be bound by theory, the inventor believes that the H3/H4 histone genes of the humans and other mammals are not divergently transcribed. Consequently, in a preferred embodiment, the method of the invention may be used to specifically amplify non-coding sequence between the H3 and H4 histone gene pair of eukaryotic organisms in clinical samples without amplifying the non-coding region from the subjects DNA. This embodiment has the advantage that background amplification from the non-coding region of the subjects histone genes is reduced or eliminated, resulting in greater sensitivity of detection.

Examples of diseases that may be diagnosed using the method of the invention include histoplasmosis, coccidioidomycosis, blastomycosis, paracoccidioidomycosis, sporotrichosis, cryptococcosis, candidiasis, aspergillosis, mucormycosis, mycetoma, chromomycosis, phaeohyphomycosis.

The method of the invention may be used to identify a purified or isolated eukaryotic organism provided the organism has a pair of divergently transcribed histone genes. For example, the non-coding region between the H2a-H2b and/or H3-H4 histone gene pairs may be amplified and the resulting probe hybridised to reference nucleic acid as discussed above to identify the species of

purified or isolated organism.

The invention will now be further described by way of reference only to the following non-limiting examples. It should be understood, however, that the examples following are illustrative only, and should not be taken in any way as a restriction on the generality of the invention described above.

Example 1: Amplification of the non-coding sequence between the coding sequence of the H2a/H2b gene pair from various species of yeast

Introduction

The aim of this experiment was to demonstrate the ability of a first and second primer complementary to the coding sequence of the H2a/H2b histone gene pair to amplify the non-coding sequence between the coding sequence from a number of species of yeast.

Nucleic acid isolation

Nucleic acid was isolated from the yeast species *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii* and *Torulaspora delbrueckii*. Yeast were grown on agar plates containing 2% glucose, 1% peptone, 0.5% yeast extract, 2% agar for 48% at 30°C. Yeast colonies were scrapped from the plate, resuspended in 1 ml of protoplast buffer (1 M sorbitol, 40 mM cultures were vortexed for one minute, incubated at 95°C for 5 minutes prior to vortexing for an additional two minutes. The cell suspension was centrifuged for 30 seconds, and the supernatant transferred to a 1.5 ml microfuge tube. The DNA in the supernatant was precipitated in ethanol, resuspended in 50 µl of TE. The DNA was then purified using a Qiagen QIAquick PCR purification kit according to the manufacturers instructions, and resuspended in 50 µl of EB buffer.

Polymerase Chain Reaction

Primer H2AR1 (SEQ ID NO. 26) and H2BR (SEQ ID NO. 20) were used as first and second primers, respectively, to amplify the non-coding sequence between the coding sequences of the H2a/H2b histone gene pair from the yeast species *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii* and *Torulaspora delbrueckii* using polymerase chain reaction.

PCR reactions were performed according to the following conditions using a Perkin Elmer thermal cycler (model 480): template DNA was added to a final concentration of 1.75 ng/ml in a buffer composed of 1X Amplitaq gold PCR buffer, 5 mM magnesium chloride, 0.48 mM DMTP's, and 0.25 U/ml Amplitaq gold enzyme. Forward and reverse primers were added at a final concentration of 2μg/ml. After a 10 minute 95°C activation of the Amplitaq gold DNA polymerase (Perkin Elmer), 35 PCR cycles (95°C 1 minute, 55°C 2 minutes and 72°C 1 minute) were performed.

Sequence analysis

Following PCR, reaction mixtures were separated by gel electrophoresis in a 0.8% agarose gel. In both *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*, two bands (corresponding to two probes) were amplified using the primers. In the case of *Torulaspora delbrueckii*, only one band (corresponding to one probe) was amplified. The complete sequence of the amplified region was obtained from the two bands of *Saccharomyces cerevisiae* (large and small) (SEQ ID NO. 235 and 236) whilst one band was completely sequenced from *T. delbrueckii* (SEQ ID NO. 237) and *Z. rouxii* (SEQ ID NO. 238). Translation start sites are shown in bold.

The sequences from each band were aligned as shown in Figure 1. As can be seen from Figure 1, alignment of the structural gene sequences shows that the amplified regions are highly homologous within the coding regions, but share limited if any homology between the translation start sites (shown in bold). It is therefore possible to design

primers which amplify the region between the translation start sites and this amplified region may be used to differentiate species. Alternatively, the amplified region could be directly labelled and used to probe a filter, microarray or any other such devise available to those skilled in the art to determine whether a particular species was present within the sample.

Conclusions

Primers complementary to the coding sequence of the divergently transcribed histone gene pair H2a/H2b are capable of amplifying the non-coding sequence between the coding sequence of the H2a/H2b histone gene pair from a plurality of yeast species. The non-coding regions between the coding regions differ between the different yeast species, while the coding sequence is highly conserved. The yeast species *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* possess two sets of H2a/H2b gene pairs, while the yeast species *T. delbrueckii* appears to posses one H2a/H2b gene pair.

Amplified region from *Saccharomyces cerevisiae* (SEQ ID NO. 235)

Large

CTTTGGGCGTAGTTACCTCTTCTTAGCAATCTGTGCACTCTACCGACTGGGAATGTCAAACCAGCCTTAGC
AGATCTAGATTGAGAAGCTTTAGCAGCTGAACCAGCTTTACCACCTTTACCACCGGACATTTTATATTTTA
TATGTATGAAATTTGTTTGTTTTGAAGTTGTTTATTCACTGAGAAATAACCAAATCCGTATGATGATGTAG
TATCAAGAAGAGAAGTACAGATTGGAAGTAAATAGATGATGGTTCAACAAGACCAGAAAATCTACAAGCTG
ATTAGGAGTCTTATTTATATATTTTTTAGGTCAAGACTTATTGCTAGTATTTACGATCCACTGGCTGGCTT
CGTGAACGGGGAAGGGGGTGAGAAAAGATTTTGAAATCAACAAAGTGGGCAATAACAAATAACAGCATGAG
AAACCACATATCTCTACGGGCGTTTCTTCAACAACGACGAGTTAACTATTGTGCTCTTTTTTTGAGCCACC
AAATACACTCCATTCCAATAGCTTCGCACAGTGAGGCGAAAATTTTGGAACAGCGCTAATGAATTATTTGT
GAGCTCGGCGAGTTCAAATTTGAAGAAAACGCGGTTGGGTCGTTAACTATGGTTAGACGCTCAATGTCGCC
CGAAAGGGAAGGCTGTTCTCACTTTTTCGCGCGTTGCACCCTTTCTTCCGCGAAAAAATGAGAACGATGGA
TTTAAAATCAAGAGAATTGGCCTTAGTAGTGGCAAATACTACCTTGGTTGGTTATCTTGTAACGATTGGTA
AGAAAGGGGCATCTCTGTTTTCTTGATGTATATAAACAACATGATTTGATCATCTCAGATGGTCAGATTTA
TTAAAGACGTTTCTCTTTCCGCATTTTCGATTATTGTTATATTAAATTTATCCTATATAGACAAGTCAAAC

CACAAATAAACCATACACACATACA**ATG**TCTGCTAAAGCCGAAAAGAAACCAGCCTCCAAAGCCCCAGCTG
AAAAGAAACCAGCCGCTAAAAAGACTTCCACTTCCACTGATGGTAAGAAGAGAAGCAAGGCTAGAAAGGAA
ACATACTCTTCTTACATTTACAAAG

Small (SEQ ID NO. 236)

TGTTAAACCAGCTTTAGCAGATCTAGATTGAGAAGCTTTAGCAGCTGAACCAGCTTTACCACCTTTACCAC
CGGA**CAT**TATATATTAAATTTGCTCTTGTTCTGTACTTTCCTAATTCTTATGTAAAAAGACAAGAATTTAT
GATACTATTTAATAACAAAAAACTACCTAAGAAAAGCATCATGCAGTCGAAATTGAAATCGAAAAGTAAAA
CTTTAACGGAACATGTTTGAAATTCTAAGAAAGCATACATCTTCATCCCTTATATATAGAGTTATGTTTGA
TATTAGTAGTCATGTTGTAATCTCTGGCCTAAGTATACGTAACGAAAATGGTAGCACGTCGCGTTTATGGC
CCCCAGGTTAATGTGTTCTCTGAAATTCGCATCACTTTGAGAAATAATGGGAACACCTTACGCGTGAGCTG
TGCCCACCGCTTCGCCTAATAAAGCGGTGTTCTCAAAATTTCTCCCCGTTTTCAGGATCACGAGCGCCATC
TAGTTCTGGTAAAATCGCGCTTACAAGAACAAAGAAAAGAAACATCGCGTAATGCAACAGTGAGACACTTG
CCGTCATATATAAGGTTTTGGATCAGTAACCGTTATTTGAGCATAACACAGGTTTTTAAATATATTATTAT
ATATCATGGTATATGTGTAAAATTTTTTTGCTGACTGGTTTTGTTTATTTATTTAGCTTTTTAAAAATTTT
ACTTTCTTCTTGTTAATTTTTTCTGATTGCTCTATACTCAAACCAACAACAACTTACTCTACAACTA**ATG**T
CCTCTGCCGCCGAAAAGAAACCAGCTTCCAAAGCTCCAGCTGAAAAGAAGCCAGCTGCCAAGAAAACATCA
ACCTCCGTCGATGGTAAGAAGAGATCTAAGGTTAGAAAGGAGACCTATTCCTCTTATATTTACAAAGTTTT
GAAGCAAACTCACCCAGACACTGGTATTTCCCAGAAGTCTATGTCTATTTTGAACTCTTTCGTTAACGATA
TCTTTGAAAGAATTGCTACTGAAGCTTCTAAATTGGCCGCTTATAACAAGAAATCCACTATTTCTGCTAGA
GAAATCCAAACAGCCGTTAGATTGATCTTACCTGGTGAATTGGCTAAACATGCCGTCTCCGAAGGTACTAG
GGCTGTTACCAAATACTCCTCCTCTACTCAAGCCTAAGTCACTCACTAGGTATTGTGATTTAG

Sequence of amplified region from *Torulaspora delbrueckii* (SEQ ID NO. 237)

GGTGNGTTGTACATGTAAGAAGAATAAGTTTCCTTTCTGACCTTACTTCTCTTCTTGTNACTGNCAA
CGGAAGAAGCAGTCTTCTTAGCAGCTGGTTTCTTCTCAGCTGGGGCTTNGGAAGCAGGTTTCTTTTC
AGCTTTGGCAGA**CAT**TATTNGNTTTAGTTTGTNTTGATTTGTTTAGTATTTTTTATTTAGAAAAATT
AAGAAACTCTCAATACCTCCAATAAGGCCTCCTTTTATATGAAATAGTTCAATCACTTCAAGATCAA
GTCAATTAATGCTTAACTATGGGCAGTCATAAGATGTGTTCTCATAACTTCGCTAATAAACCTATCT
TAACTATGTATAAACTGAAGGCGAAATTTTCAGCACGGAACGATTCCGCATTTGGCAATCGCGCTGT
CTATCCATAGTTAAGCGAAATCAATTTTTTGTTTGAAAAATGAACTCGTCGCGATCGGAAAGAAACC
GACTTCAACGTTCCAGGTTTTTCACAGCACACGGTGAAGGTTCTGGTCCAAGGGGGGCGCGCCAAAA
GGCCTAGTCTTGTAAAGTCAAGGACGAAGCGTATATAAACGGGCCTTATAATCTATCCAGAGGCAAC

ATCTCCTTCTCAACTCACACAACACAAACAGAATACATGTCAGGTGGTAAAGGTGGTAAAGCAGGTT
CTGCCGCAAAGGCTTCTCAATCCAGATCAGCTAAGGCTGGTCTAACTTTCCCAGTCGGTAGAGTTCA
CAGACTGTTAAGAAAAGGTAACTACGGTCAAA

Sequence of amplified region from *Zygosaccharomyces rouxii* (SEQ ID NO. 238)

CTTTGACCATAGTTACCCTTTCTCAAAAGTCTGTGAACTCTACCAACTGGGAAGGTCAAACCTGCCTTAGC
AGATCTTGATTGAGAAGCCTTAGCTGCGGAACCTGCTTTACCACCTTTACCACCGGACATTGTTATATTTG
TTGTATTTGTGATTGTTGTTTGTGTTGTAGAAAAGTAAGAGTAAAGAAATCATGTATCTCACCAGTAACCT
TCCTCCTTTTATACCGTTACCTTTCCCTTAGGAATTCGCCACTGACTGAATCCCCTGTTCCATTTTTTTCT
CACCCGGTGTGAAATAACTGGAACGTTGGAATTCGGTTCCAAGAACTATTAAAAGTGACCGTAGTTGATGA
AAAATTAATTTTCTCTTAACTATAGGTATCGGTCCGTACTACCGCATTCGGTAACTCCATTCTGAAAAATT
CACACCCAGGGGCGAAAAAATAGGTACGGTATTTAATTTAACAAAGGATTCCAAAAGGTATAAAAAGAGAC
AAGGTATTGGTATAGAACACGATAATCGATCCTTACAACTACCCACCCGGTGATCCATTANATTCAAATCA
AACACAACACATACAAATCAAAATGTCTGCTAAAGCTAAAAGAAACCCGCTTCCAAGGCCCCAGCTGAAAA
GAAGCCAGCTGCCAAGAAAACCGCATCTTCTGTCGAAGGTAAGGGTAAGAAGAACAAGGCAAGAAAGGAGA
CCTACTCCTCTTACATTTACAACNCACCN

Example 2: Speciation of three yeast strains using a combination of general and specific primers.

Saccharomyces cerevisiae, Kluyveromyces marxianus and *Zygosaccharomyces rouxii* were grown on agar containing 2% glucose, 1% peptone and 0.5% yeast extract. A single colony from each was resuspended in 1 ml sterile distilled water. Five microlitres of the cell suspension was added to a PCR reaction mixture containing:

5µl of $MgCl_2$ (25mM)
5 µl of PCR buffer
0.625 µl of 25mM dNTP's
1 µl of primer mix 1
1 µl of primer mix 2
0.25 µl of amplitaq gold
32.125 µl of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 primers H2AR1 (SEQ ID NO. 26) and H2ACere (SEQ ID NO. 46), each of which was at a concentration of 100ng/µl in water.

Primer mix 2 was made by combining in a ratio of 1:1 primers H2BRCere (SEQ ID NO. 47) and H2BRYEAST (SEQ ID. NO 27), each of which was at a concentration of 100ng/µl in water.

The reaction mixes were subject to the following PCR protocol:

Step 1: Denaturation of template and activation of Amplitaq gold:

95°C for 15 min

Step 2: Amplification of histone regions:

35 cycles, each composed of

95°C 1 min
50°C 1 min
72°C 1 min 45 sec

After the PCR cycles were complete, the DNA was diluted 1:100 in water and used as a template for a second PCR reaction which was performed as follows:

5ml of the 1:100 dilution of the first PCR reaction
5µl of $MgCl_2$ (25mM)
5 µl of PCR buffer
0.625 µl of 25mM dNTP's
1 µl of primer species specific primer
1 µl of primer species specific primer
0.25 µl of amplitaq gold
32.125 µl of sterile distilled water

Nine reactions were performed as follows:

1. *S. cerevisiae* PCR product with *S. cerevisiae* specific primers.
2. *S. cerevisiae* PCR product with *Z. rouxii* specific primers.
3. *S. cerevisiae* PCR product with *K. marxianus* specific primers.
4. *Z. rouxii* PCR product with *S. cerevisiae* specific primers.
5. *Z. rouxii* PCR product with *Z. rouxii* specific primers.
6. *Z. rouxii* PCR product with *K. marxianus* specific primers.
7. *K. marxianus* PCR product with *S. cerevisiae* specific primers.
8. *K. marxianus* PCR product with *Z. rouxii* specific primers.
9. *K. marxianus* PCR product with *K. marxianus* specific primers.

The primer sequences were as follows:

K. marxianus:

H2bL GAGAAAACCCCACTTACAATATGCG (SEQ ID NO.171)
H2aL CAGGAGGGTACAACTAGCTACCACTC (SEQ ID NO. 172)

S. cerevisiae:

H2bs GACGGCAAGTGTCTCACTGTTGCATTACG (SEQ ID NO.181)
H2aS CGAAAATGGTAGCACGTCGCGTTTATGG (SEQ ID NO.182)

Z. rouxii:

H2b GGATCACCGGGTGGGTAGTTGTAAGG (SEQ ID NO.191)
H2a CTCACCCGGTGTGAAATAACTGGAACG(SEQ ID NO.192)

The reaction mixes were subjected to the following PCR protocol:

Step 1: Denaturation of template and activation of Amplitaq gold

95°C for 15 min

Step 2: Amplification of histone regions

25 cycles, each composed of
95°C 1 min
60°C 1 min
72°C 1 min

After completion of the PCR reaction, the samples were purified using a Quiagen PCR purification kit, and the PCR products resuspended in 100µl of buffer EB. 0.5µl of a 1:1000 Dilution of the SYTO BC dye (Molecular probes B7277) was added to each of the nine samples, prior to transfer to a 96 well microtitre tray. The microtitre tray was scanned and the image captured using a Typhoon 9410 scanner using 488nm light for excitation, and a 530 filter for detection.

As shown in Figure 2, the kit successfully identified each of the different yeast species.

<u>Example 3. Amplification of polymorphic non-coding region between the H3-H4 histone gene pair from yeast DNA</u>

Chromosomal DNA was obtained from the following yeast species:

Candida albicans (CBS562), *Candida dubliniensis* (CBS8501), *Candida glabrata* (CBS138), *Candida parapsilosis* (99-62400), *Candida lipolytica* (CBS566), *Candida krusei* (CBS573), *Candida norvegensis* (CBS1922), *Candida lusitaniae* (CBS4413), *Cryptococcus neoformans* VG III (WM161), *Candida krusei* (FRR 1307), *Candida lusitaniae* (FRR4507), *Candida multigemmis* (FRR4706), *Candida parapsilosis* (FRR4940), *Candida tropicalis* (FRR1284), *Candida utilis* (FRR1777), *Debaryomyces hansenii* (FRR 2577), *Dekkera anomala* (FRR4647), *Dekkera bruxellensis* (FRR4650), *Endomyces fibuliger* (FRR 4359), *Geotrichum*

candidum (FRR 2424), *Kloeckera valbyensis* (FRR2168), *Kluyveromyces marxianus* (FRR5155), *Leucosporidium scottii*(FRR5393), *Metschnikowia pulcherrima* (FRR4798), *Pichia anomala* (FRR4420), *Pichia guillermondii* (FRR4711), *Pichia membranaefaciens* (FRR4425), *Rhodotorula glutinis* (FRR4522), *Rhodotorula mucilaginosa* (FRR4309), *Rhodotorula rubra* (FRR2406), *Saccharomyces cerevisiae* (AEM 27), *Schizosaccharomyces pombe* (FRR2208), *Torulaspora delbrueckii* (FRR4508), *Torulopsis holmii* (FRR1300),*Yarrowia lipolytica* (FRR1728), *Zygosaccharomyces bailii*(FRR 1292), *Zygosaccharomyces bisporus* (FRR2018), *Zygosaccharomyces rouxii* (FRR3667).

To obtain chromosomal DNA for each species, cultures of each of the above yeast species were grown on GYP (glucose 2%, Yeast extract 0.5%, Peptone 1%, agar 2%) agar plates for 2 days at 30°C. Cells were harvested from the plate and resuspended in 1 ml of buffer 1 (1 M sorbitol, 40 mM KH_2PO_4, 1 mM KH_2PO_4 pH 7.5) in a 2 ml screw cap microfuge tube. The cells were then heat treated to 95°C for 15 minutes. Following heat treatment, the tube was half filled with glass beads (SIGMA, acid-washed 425-600 microns), placed in a FastPrep FP120 (Bio101 SAVANT) and beaten twice on speed 5 for 20s. The tubes were then centrifuged for 1 min to pellet the cell debris and glass beads. 500 ul of the supernatant was removed and heated to 95 degrees celcius for 5 minutes. The heat treated sample was centrifuged in a microfuge at full speed for 1 minute. 400 ul of sample was transferred to a new tube, followed by addition of 40 ul of 5 M potassium acetate and DNA was precipitated by addition of 880 ul of ethanol and chilling to -20 degrees celcius. Precipitated DNA was pelleted by centrifugation, and the supernatant removed.

The precipitated DNA was resuspended in 100 ul of buffer EB (from Qiagen QIAquick PCR purification kit) and processed according to the QIAquick PCR purification protocol. The DNA was finally eluted in 50 ul of EB buffer.

The chromosomal DNA was diluted 1:10, and 5 ul was used in the PCR reaction as described below.
5 ul of chromosomal DNA from a single yeast species was added to a PCR reaction mix containing the following:

5 ml of $MgCl_2$ (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH3ID CTTGCAGTTTGYTTRGTDCKNGCC (SEQ ID NO. 22)
lusitH3ID CTTGCGGTTTGTTTGGTTCTAGCC (SEQ ID NO. 239)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH4ID CCTTTTCCACCTTTWCCNCTDCCRG (SEQ ID NO. 24)
multiH4ID CCTTTTCCTCCTTTTCCTCTACCAG (SEQ ID NO. 240)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplitaq gold

95^0C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^0C 1 min
55^0C 1 min
72^0C 1 min 45 sec

10 ul of each of the PCR samples was electrophoresed on a 2% agarose gel to examine the PCR products. The results of the electrophoresis are illustrated in Figure 3. Lane designations are as follows: M = BRL 1kb marker, 1= *Candida albicans* (CBS562), 2 = *Candida dubliniensis* (CBS8501), 3 = *Candida glabrata* (CBS138), 4 = *Candida parapsilosis* (99-62400), 5 = *Candida lipolytica* (CBS566), 6 = *Candida krusei* (CBS573), 7 = *Candida norvegensis* (CBS1922), 8 = *Candida lusitaniae* (CBS4413), 9 = *Cryptococcus neoformans* VG III (WM161), 10 = *Candida krusei* (FRR 1307), 11 = *Candida lusitaniae* (FRR4507), 12 = *Candida multigemmis* (FRR4706), 13 = *Candida parapsilosis* (FRR4940), 14 = *Candida tropicalis* (FRR1284), 15 = *Candida utilis* (FRR1777), 16 = *Debaryomyces hansenii* (FRR 2577), 17 = *Dekkera anomala* (FRR4647), 18 = *Dekkera bruxellensis* (FRR4650), 19 = *Endomyces fibuliger* (FRR 4359), 20 = *Geotrichum candidum* (FRR 2424), 21 = *Kloeckera valbyensis* (FRR2168), 22 = *Kluyveromyces marxianus* (FRR5155), 23 = *Leucosporidium scottii* (FRR5393), 24 = *Metschnikowia pulcherrima* (FRR4798), 25 = *Pichia anomala* (FRR4420), 26 = *Pichia guillermondii* (FRR4711), 27 = *Pichia*

membranaefaciens (FRR4425), 28 = *Rhodotorula glutinis* (FRR4522), M = BRL 1kb marker, 29 = *Rhodotorula mucilaginosa* (FRR4309), 30 = *Rhodotorula rubra* (FRR2406), 31 = *Saccharomyces cerevisiae* (AEM 27), 32= *Schizosaccharomyces pombe* (FRR2208), 33 = *Torulaspora delbrueckii* (FRR4508), 34 = *Torulopsis holmii* (FRR1300), 35 = *Yarrowia lipolytica* (FRR1728), 36 = *Zygosaccharomyces bailii* (FRR 1292), 37 = *Zygosaccharomyces bisporus* (FRR2018), 38 = *Zygosaccharomyces rouxii* (FRR3667).

It can be seen that the primer mixes produced a PCR product in each of the yeast species tested. Sequencing of selected bands confirmed that they were derived from divergent histone loci.

Example 4 Amplification of the non-coding region between the H3-H4 histone gene pair from fungal species.

Chromosomal DNA was obtained from each of the following fungal species:

Aspergillus ochraecus (FRR2360), *Aspergillus fumigatus* (FRR4970), *Penicillium crustosum* (FRR3315), *Rhizopus stolonifer* (FRR2053)

Chromosomal DNA from each of the above species was obtained as described in Example 3.

The resulting chromosomal DNA from each species was diluted 1:10, and 5 ul was used in the PCR reaction as described below.
5 ul of chromosomal DNA was added to a PCR reaction mix containing

5 ul of MgCL2 (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's

1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water

H4FIL AATAGCGGGYTTNGTDATNCCYTG (SEQ ID NO. 28)
H4 A fumigat AATAGCGGGCTTGGTGATACCCTG (SEQ ID NO. 241)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water

H3FIL GTAGCGACGAATYTCNCGNAGNGC (SEQ ID NO. 38)
H3 A fumigat GTAGCGACGAATCTCACGGAGAGC (SEQ ID NO. 242)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplitaq gold

95^0C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^0C 1 min
50^0C 1 min
72^0C 1 min 45 sec

10 ul of the PCR sample was electrophoresed on a 2% agarose gel to confirm that the histone PCR primers had amplified the fragments in the expected size range (1.2 kb for *A. fumigatus*). The results of the electrophoresis are shown in Figure 4. Lane designations are as follows: M = BRL 1kb marker 1= *Aspergillus ochraecus* (FRR2360), 2 = *Aspergillus fumigatus* (FRR4970), 3 = *Penicillium crustosum* (FRR3315), 4 = *Rhizopus stolonifer* (FRR2053) M = BRL 1kb marker

It can be seen that the primer mixes produced PCR products from all 4 species tested.

Example 5 Amplification of polymorphic non-coding region between the H2A-H2B histone gene pair from yeast

Chromsomal DNA was extracted from the following yeast: *Kloeckera apiculata* (YPG1079), *Kluyveromyces marxianus* (YPG974), *Metschnikowia pulcherrima* (YPG1093), *Pichia guillermondii* (FRR4711), *Saccharomyces cerevisiae* (AEM 27), *Torulaspora delbrueckii* (YPG477), *Zygosaccharomyces bailii* (YPG478) *Zygosaccharomyces rouxii* (YPG476)

The above yeast cultures were grown on GYP (glucose 2%, Yeast extract 0.5%, Peptone 1%, agar 2%) agar plates for 2 days at 30°C. Chromosomal DNA was then extracted as described for Example 3 above.

The chromosomal DNA was diluted 1:10, and 5 ul was added to a PCR reaction mix containing the following:

5 ul of $MgCl_2$ (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold

32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water

H2AR1 GYTARRTAARCKGGAGCACC (SEQ ID NO. 26)
H2ACere GTTAGATAGACTGGAGCACC (SEQ ID NO. 46)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water

H2BRCere CCTGTGTCTGGGTGAGTTTG (SEQ ID NO. 47)
H2BRYEAST CCAGTGTCAGGYTGNGTYTG (SEQ ID NO. 27)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplitaq gold

95^{0}C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^{0}C 1 min
50^{0}C 1 min
72^{0}C 1 min 45 sec

10 ul of the PCR sample was electrophoresed on a 2% agarose gel to examine the PCR products. The results of electrophoresis are shown in Figure 5. Lane designations are as follows: M = BRL 1kb marker, 1 = *Saccharomyces cerevisiae* (AEM 27), 2 = *Zygosaccharomyces rouxii* (YPG476), 3 = *Zygosaccharomyces bailii* (YPG478), 4

= *Torulaspora delbrueckii* (YPG477), 5 = *Kluyveromyces marxianus* (YPG974), 6 = *Metschnikowia pulcherrima* (YPG1093), 7 = *Kloeckera apiculata* (YPG1079), 8 = *Pichia guillermondii* (FRR4711).

It can be seen that PCR products were produced in all cases.

Cloning and sequencing of selected bands confirmed that they were derived from divergent histone loci and little if any sequence homology was detected between the species within the non-coding region.

<u>Example 6. Selective amplification of yeast DNA from a mixture of human and yeast cells</u>

Three PCR reactions were set up to determine whether the non-coding region between yeast H3 and H4 histone gene pairs could be specifically amplified in a sample containing human cells. PCR reaction 1 contained pure yeast cells derived from an oral thrush infection. PCR reaction 2 contained pure yeast cells derived from an oral thrush and buccal cells mixed together. PCR reaction 3 contained pure buccal cells. PCR reactions were set up as follows:

5 ul of cells were added to a PCR reaction mix containing the following:

5 ul of PCR buffer (Roche PCR DIG probe synthesis kit vial 3)
5 ul of dNTPs (Roche PCR DIG probe synthesis kit vial 4)
1 ul of primer mix 1
1ul of primer mix 2
0.75 ul of enzyme mix (Roche PCR DIG probe synthesis kit vial 1)
32.25 ul of sterile distilled water.

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH3ID CTTGCAGTTTGYTTRGTDCKNGCC(SEQ ID NO. 22)
lusitH3ID CTTGCGGTTTGTTTGGTTCTAGCC (SEQ ID NO. 239)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH4ID CCTTTTCCACCTTTWCCNCTDCCRG (SEQ ID NO. 24)
multiH4ID CCTTTTCCTCCTTTTCCTCTACCAG (SEQ ID NO. 240)

Step 1 Denaturation of template 95^{0}C for 3 min

Step 2 Amplification of Histone H3-H4 promoter regions

35 cycles, each composed of

95^{0}C 45 seconds
55^{0}C 1 minute
72^{0}C 1 minute

10 ul of the PCR sample was electrophoresed on a 2% agarose gel to examine the PCR products. The results of electrophoresis are shown in Figure 6. Lane designations are as follows: M = BRL 1kb marker, 1 = yeast cells only, 2 = yeast cells + buccal cells, 3 = buccal cells only. It can be seen that an amplification product was obtained from the samples containing yeast, and yeast and buccal cells, but not from the sample containing buccal cells alone. This indicates that the primers designed to amplify the non-coding region of the H3 H4 locus selectively amplified yeast DNA even in the presence of human cells.

Example 7. Differentiation of yeast species using RFLP analysis of non-coding region between the H3-H4 histone gene pair.

Chromosomal DNA was obtained from the following yeast species:
Candida albicans (CBS562), *Candida dubliniensis* (CBS8501), *Candida tropicalis* (FRR1284), *Candida krusei* (CBS573), *Candida multigemmis* (FRR4706), *Candida parapsilosis* (99-62400), *Candida lipolytica* (CBS566), *Candida norvegensis* (CBS1922), *Candida glabrata* (CBS138), *Saccharomyces cerevisiae* (AEM 27), *Cryptococcus neoformans* VG III (WM161), *Candida lusitaniae* (CBS4413), *Rhodotorula mucilaginosa* (FRR4309), *Rhodotorula rubra* (FRR2406).

Chromosomal DNA was extracted from the above species using the method described in Example 3.

The chromosomal DNA was diluted 1:10, and 5 ul was used in the PCR reaction as described below.
5 ul of chromosomal DNA was added to a PCR reaction mix containing the following:

5 ul of $MgCL_2$ (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH3ID	CTTGCAGTTTGYTTRGTDCKNGCC(SEQ ID NO. 22)
lusitH3ID	CTTGCGGTTTGTTTGGTTCTAGCC (SEQ ID NO. 239)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH4ID	CCTTTTCCACCTTTWCCNCTDCCRG	(SEQ ID NO. 24)
multiH4ID	CCTTTTCCTCCTTTTCCTCTACCAG	(SEQ ID NO. 240)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplitaq gold

95^{0}C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^{0}C 1 min
55^{0}C 1 min
72^{0}C 1 min 45 sec

Following PCR amplification, 5 ul of amplified DNA was added to a restriction endonuclease digestion mix containing:

10x Buffer	2 ul	
Hae III	1 ul	(Roche diagnostics)
water	12 ul	

and incubated at 37°C for 1 hour prior to running a 10 ul aliqout of each digest on a 3% agarose gel. The results of electrophoresis on the agarose gel are shown in Figure 7. Lane designations are as follows: M = BRL 1 kb ladder, 1 = *Candida albicans* (CBS562), 2 = *Candida dubliniensis* (CBS8501), 3= *Candida tropicalis* (FRR1284), 4 = *Candida*

krusei (CBS573),), 5 = *Candida multigemmis* (FRR4706), 6 = *Candida parapsilosis* (99-62400), 7 = *Candida lipolytica* (CBS566), 8 = *Candida norvegensis* (CBS1922), 9 = *Candida glabrata* (CBS138), 10 = *Saccharomyces cerevisiae* (AEM 27), 11 = *Candida lusitaniae* (CBS4413), 12 = *Rhodotorula mucilaginosa* (FRR4309), 13 = *Rhodotorula rubra* (FRR2406).

It can be seen from Figure 7 that the RFLP pattern obtained from the PCR product for each species allows differentiation between the species.

Example 8. Identification of yeast species by sequence analysis of amplified non-coding region between histone gene pairs.

PCR amplification of the non-coding region between the H3-H4 and H2A-H2B histone gene pairs of a strain of yeast suspected to be *Saccharomyces cerevisiae* was performed. One clone of the H3-H4 non-coding region and two clones of the H2A-H2B non-coding region were cloned into the TA cloning vector according to the manufacturers instructions (Invitrogen). Each region was completely sequenced and the non coding regions examined (shown below). The results of the sequencing are shown in Figure 8. Comparison of the sequenced regions with the Genbank database using the BLAST program (National Centre for Biotechnology Information) indicated these sequences showed significant (>95%) homology only with the sequences of the published *S. cerevisiae* genome, indicating that the species tested was *S. cerevisiae*.

Example 9. Identification of yeast to species level using nested PCR on non-coding region between the H3-H4 histone gene pair directly from cells.

A swab was taken from a patient with oral thrush (suspected to be *C. albicans*) and the sample was plated

out onto GYPagar plates. After incubation a loopful of cells was resuspended in 1 ml sterile distilled water.

5 ul of the cell suspension was added to a PCR reaction mix containing:

5 ul of $MgCL_2$ (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH3ID CTTGCAGTTTGYTTRGTDCKNGCC (SEQ ID NO. 22)
lusitH3ID CTTGCGGTTTGTTTGGTTCTAGCC (SEQ ID NO. 239)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH4ID CCTTTTCCACCTTTWCCNCTDCCRG (SEQ ID NO. 24)
multiH4ID CCTTTTCCTCCTTTTCCTCTACCAG (SEQ ID NO. 240)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplittaq gold

95^{0}C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^0C 45 seconds
55^0C 1 minute
72^0C 1 minute

10 ul of the PCR reaction was run on a 1% gel to confirm amplification of the non-coding region from between the H3-H4 histone gene pair from the unknown sample. The results of the electrophoresis are shown in Figure 9a and confirm that the expected region was amplified.

The PCR amplified DNA (a sample of which is shown in Figure 9a) was diluted 1:100 in water and used as a template for a second PCR reaction which was performed as follows:

5 ul of the 1:100 dilution of the first PCR reaction

5 ul of $MgCL_2$ (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer species specific primer
1 ul of primer species specific primer
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Three reactions were performed

1 Unknown yeast PCR product with *C. albicans* specific primers
2 Unknown yeast PCR product with *C. glabrata* specific primers
3 Unknown yeast PCR product with *S. cerevisiae* specific primers
4 No DNA control

The primer sequences were determined by examining the published sequence of the H3-H4 intergenic region of each of the three different species.

S. cerevisiae specific primers:

CGCACAAACACGTATGTATCTAGCCG (SEQ ID NO. 215)
CTTAATGCGAAGTGCTCGGAACGGAC (SEQ ID NO. 216)

C. albicans specific primers:

GTTTGTGCCTGGTGCGAAATGG (SEQ ID NO. 217)
GGGAGTAAAGGAAGAAGAGTTC (SEQ ID NO. 218)

C. glabrata specific primers:

GTAATTTGCCACAACCACCAAGCACC (SEQ ID NO. 233)
GTTGGACGGTATGCGTATTTCAGGCG (SEQ ID NO. 234)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplittaq gold

95^0C for 15 min

Step 2 Amplification of Histone regions

25 cycles, each composed of

95^0C 45 seconds
60^0C 1 minute
72^0C 1 minute

10 ul of the PCR products were examined by gel electrophoresis. The results of the electrophoresis are shown in Figure 9b. Lane designations are as follows: 1= C. albicans specific primers, 2 = C. glabrata specific

primers, 3 = S. cerevisiae specific primers, 4 = No DNA control, M = BRL 1kb marker.

It can be seen that the only PCR product obtained is in lane 1 indicating that the unknown yeast derived from a case of oral thrush is *Candida albicans*.

Example 10. Characterisation of yeast to species level using the non-coding region between the H3-H4 histone gene pair to probe a filter composed of a panel of amplified DNA derived from a known assembly of yeast species.

DNA was extracted from the following cultures: *Candida albicans* (CBS562), *Candida dubliniensis* (CBS8501), *Candida glabrata* (CBS138), *Candida parapsilosis* (99-62400), *Candida lipolytica* (CBS566), *Candida krusei* (CBS573), *Candida norvegensis* (CBS1922), *Candida lusitaniae* (CBS4413), *Cryptococcus neoformans* VG III (WM161), *Candida krusei* (FRR 1307), *Candida lusitaniae* (FRR4507), *Candida multigemmis* (FRR4706), *Candida parapsilosis* (FRR4940), *Candida tropicalis* (FRR1284), *Candida utilis* (FRR1777), *Debaryomyces hansenii* (FRR 2577), *Dekkera anomala* (FRR4647), *Dekkera bruxellensis* (FRR4650), *Endomyces fibuliger* (FRR 4359), *Geotrichum candidum* (FRR 2424), *Kloeckera valbyensis* (FRR2168), *Kluyveromyces marxianus* (FRR5155), *Leucosporidium scottii*(FRR5393), *Metschnikowia pulcherrima* (FRR4798), *Pichia anomala* (FRR4420), *Pichia guillermondii* (FRR4711), *Pichia membranaefaciens* (FRR4425), *Rhodotorula glutinis* (FRR4522), *Rhodotorula mucilaginosa* (FRR4309), *Rhodotorula rubra* (FRR2406), *Saccharomyces cerevisiae* (AEM 27), *Schizosaccharomyces pombe* (FRR2208), *Torulaspora delbrueckii* (FRR4508), *Torulopsis holmii* (FRR1300)*Yarrowia lipolytica* (FRR1728), *Zygosaccharomyces bailii*(FRR 1292), *Zygosaccharomyces bisporus* (FRR2018), *Zygosaccharomyces rouxii* (FRR3667).

Chromosomal DNA from each of the above cultures was obtained using the method described in Example 3.

The chromosomal DNA was diluted 1:10, and 5 ul was used in the PCR reaction as described below.
5 ul of chromosomal DNA was added to a PCR reaction mix containing:

5 ul of MgCL2 (25 mM)
5 ul of PCR buffer
0.625 ul of 25 mM dNTP's
1 ul of primer mix 1
1 ul of primer mix 2
0.25 ul of amplitag gold
32.125 ul of sterile distilled water

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH3ID CTTGCAGTTTGYTTRGTDCKNGCC(SEQ ID NO. 22)
lusitH3ID CTTGCGGTTTGTTTGGTTCTAGCC (SEQ ID NO. 239)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

YeastH4ID CCTTTTCCACCTTTWCCNCTDCCRG (SEQ ID NO. 24)
multiH4ID CCTTTTCCTCCTTTTCCTCTACCAG (SEQ ID NO. 240)

The reaction mixes were subject to the following PCR protocol:

Step 1 Denaturation of template and activation of Amplitaq gold

95^0C for 15 min

Step 2 Amplification of Histone regions

35 cycles, each composed of

95^0C 1 min
55^0C 1 min
72^0C 1 min 45 sec

10 ul of the PCR sample was electrophoresed on a 2% agarose gel to examine the PCR products. The results of the electrophoresis are shown in Figure 10a. Lane designations are as follows: M = BRL 1kb marker, 1= *Candida albicans* (CBS562), 2 = *Candida dubliniensis* (CBS8501), 3 = *Candida glabrata* (CBS138), 4 = *Candida parapsilosis* (99-62400), 5 = *Candida lipolytica* (CBS566), 6 = *Candida krusei* (CBS573), 7 = *Candida norvegensis* (CBS1922), 8 = *Candida lusitaniae* (CBS4413), 9 = *Cryptococcus neoformans* VG III (WM161), 10 = *Candida krusei* (FRR 1307), 11 = *Candida lusitaniae* (FRR4507), 12 = *Candida multigemmis* (FRR4706), 13 = *Candida parapsilosis* (FRR4940), 14 = *Candida tropicalis* (FRR1284), 15 = *Candida utilis* (FRR1777), 16 = *Debaryomyces hansenii* (FRR 2577), 17 = *Dekkera anomala* (FRR4647), 18 = *Dekkera bruxellensis* (FRR4650), 19 = *Endomyces fibuliger* (FRR 4359), 20 = *Geotrichum candidum* (FRR 2424), 21 = *Kloeckera valbyensis* (FRR2168), 22 = *Kluyveromyces marxianus* (FRR5155), 23 = *Leucosporidium scottii* (FRR5393), 24 = *Metschnikowia pulcherrima* (FRR4798), 25 = *Pichia anomala* (FRR4420), 26 = *Pichia guillermondii* (FRR4711), 27 = *Pichia membranaefaciens* (FRR4425), 28 = *Rhodotorula glutinis* (FRR4522), M = BRL 1kb marker, 29 = *Rhodotorula mucilaginosa* (FRR4309), 30 = *Rhodotorula rubra* (FRR2406), 31 = *Saccharomyces cerevisiae* (AEM 27), 32= *Schizosaccharomyces pombe* (FRR2208), 33 = *Torulaspora*

delbrueckii (FRR4508), 34 = *Torulopsis holmii* (FRR1300), 35 = *Yarrowia lipolytica* (FRR1728), 36 = *Zygosaccharomyces bailii* (FRR 1292), 37 = *Zygosaccharomyces bisporus* (FRR2018), 38 = *Zygosaccharomyces rouxii* (FRR3667) M = BRL 1kb marker. It can be seen that a PCR product was obtained from the all the yeast species.

The amplified DNA from each of the samples was was diluted 1:2 in a NaOH/EDTA buffer to make a final concentration of 0.4M NaOH/10mM EDTA solution. The buffered DNA solution was denatured by heating to 100°C and quenched on ice prior to spotting a 1 µl aliquot onto a prewet positively charged nylon filter (Roche diagnostics).

The DNA was spotted onto the filter in the following pattern:

1A = Candida albicans (CBS562)	2A = *Candida tropicalis (FRR1284)*	3A = *Pichia guillermondii* (FRR4711)
1B = Candida dubliniensis (CBS8501	2B = *Candida utilis* (FRR1777)	3B = *Rhodotorula glutinis* (FRR4522
1C = Candida glabrata (CBS138)	2C = *Debaryomyces hansenii* (FRR2577)	3C =) *Rhodotorula mucilaginosa* (FRR4309),
1D = Candida parapsilosis (99-62400	2D = *Dekkera anomala* (FRR4647)	3D = *Rhodotorula rubra* (FRR2406),
1E = Candida lipolytica (CBS566),	2E = *Dekkera bruxellensis* (FRR4650)	3E = *Saccharomyces cerevisiae* (AEM 27),
1F = Candida krusei (CBS573),	2F = *Endomyces fibuliger* (FRR4359)	3F = *Schizosaccharomyces pombe* (FRR2208),
1G = Candida	2G =)=	3G = *Torulaspora*

norvegensis (CBS1922),	*Geotrichum candidum* (FRR2424)	*delbrueckii* (FRR4508),
1H = Candida lusitaniae (CBS4413),	2H = *Kloeckera valbyensis* (FRR2168)	3H = *Torulopsis holmii* (FRR1300),
1I = Cryptococcus neoformans (WM161),	2I = *Kluyveromyces marxianus* (FRR5155)	3I = *Yarrowia lipolytica* (FRR1728),
1J = Candida krusei (FRR 1307),	2J = *Leucosporidium scottii* (FRR5393)	3J = *Zygosaccharomyces bailii* (FRR1292),
1K = Candida lusitaniae (FRR4507),	2K = *Metschnikowia pulcherrima* (FRR4798)	3K = *Zygosaccharomyces bisporus* (FRR2018)
1L = Candida multigemmis (FRR4706),	2L = = *Pichia anomala* (FRR4420)	3L = *Zygosaccharomyces rouxii* (FRR3667)
1M = Candida parapsilosis (FRR4940),	2M = *Pichia membranaefaciens* (FRR4425)	3M = Thrush isolate

The filter, containing the non-coding region between the H3-H4 histone gene pairs from each of the reference strains of yeast, was baked at 120°C for 30 minutes.

The non-coding region between the H3-H4 histone gene pair from *Candida lusitaniae* (FRR4507) was amplified and labelled using the Roche 'PCR DIG probe Synthesis Kit' by adding 5 ul of chromosomal DNA to a reaction mix containing the following:

5 ul of PCR buffer (Roche kit vial 3)

5 ul of DIG labelled dNTPs (Roche kit vial 2)
1 ul of primer mix 1
1ul of primer mix 2
0.75 ul of enzyme mix (Roche kit vial 1)
32.25 ul of sterile distilled water.

Primer mix 1 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

H3R2CERE CTCTCAAGGCAACAGTACCTGG (SEQ ID NO. 49)
H3R2YEAS CTCTCAAGGCNACNGTNCCNGG (SEQ ID NO. 37)

Primer mix 2 was made by combining in a ratio of 1:1 the following primers, each of which was at a concentration of 100 ng/ul in water:

H4R1CERE GATAGCTGGCTTAGTGATACC (SEQ ID NO. 50)
H4R1YEAS GATAGCTGGYTTNGTNATNCC (SEQ ID NO. 44)

The PCR reaction was heated to 95°C for 3 minutes prior to performing 35 of the following PCR cycles:

94°C 1 minutes
50°C 1 minutes
72°C 1 min 45 seconds

The labeled DNA was used to probe the previously prepared filter using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche).

All buffers and reagents were as described in the Roche kit.

Hybridization and signal detection were performed by heating 2 ul of the DIG labelled probe DNA to 100°C, and adding it directly to 5 mls of prehybridization buffer

that was also heated to 100°C. The denatured probe and hybridization buffer was added to the filter and incubated at 42°C for 30 minutes. The filter was washed five times in 2XSSC, prior to washing three times in a stringent washing buffer heated to 68°C. The stringent washing buffer was removed and the filter quickly rinsed twice in 10 ml washing buffer. The filter was blocked for 10 minutes in a blocking buffer prior to the addition of a 1:10000 dilution of the DIG antibody in blocking buffer. After 25 minutes, the filter was rinsed quickly in two 10 ml washes of washing buffer, then three quick rinses in 10 ml detection buffer prior to the addition of 5 ml NBT colour development reagent. After three minutes the colour reaction was complete and the reaction stopped by washing the filter in water. The results obtained are shown in Figure 10b.

It can be seen that two well-developed spots appeared on the filter at positions H1 (corresponding to *Candida lusitaniae* (CBS4413)) and K1 (corresponding to organism used to generate the probe (*Candida lusitaniae* (FRR4507))). Only a low level of background cross hybridisation was observed with some other species, confirming the ability of this test to rapidly determine the identity of a yeast species from which a divergently arranged histone promoter can be amplified.

CLAIMS:

1. A method for detecting a eukaryotic species in a sample comprising the steps of:

(i) contacting a first primer complementary to the coding sequence of a first histone gene of a pair of divergently transcribed histone genes located in said sample and amplifying at least a segment of a non-coding sequence located between and contiguous with the coding sequence of the first and a second histone gene of the pair to produce a probe substantially complementary to said non-coding sequence; and

(ii) comparing the probe with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species thereby detecting whether said species is present in said sample.

2. The method of claim 1 further comprising the step of providing a second primer complementary to the sequence of the second strand of said coding sequence of the second histone gene, and contacting said second primer with said nucleic acid molecules.

3. The method of claim 1 wherein the probe is compared to the one or more reference nucleic acids by hybridising the probe with the one or more reference nucleic acid molecules under stringent hybridisation conditions.

4. The method of claim 1 further comprising the step before step i) of extracting nucleic acid from one or more eukaryotic species in said sample.

5. The method of claim 1 wherein the first histone gene coding sequence comprises at least 15bp and the second histone gene coding sequence comprises at least 15bp.

6. The method of claim 1 wherein the pair of divergently transcribed histone genes is the H2a and H2b histone gene

pair, or the H3 and H4 histone gene pair.

7. The method of claim 1 wherein the first histone gene is selected from the group comprising H2a, H2b, H3 and H4.

8. The method of claim 2 wherein the first histone gene is H2a and the second histone gene is H2b.

9. The method of claim 1 wherein the first histone gene is H2b and the second histone gene is H2a.

10. The method of claim 1 wherein the first histone gene is H3 and the second histone gene is H4.

11. The method of claim 1 wherein the first histone gene is H4 and the second histone gene is H3.

12. The method of claim 1 wherein the probe is produced by amplifying at least a segment of the non-coding sequence by extending the first primer.

13. The method of claim 12 wherein the first primer is extended using a DNA polymerase.

14. The method of claim 2 wherein the segment of non-coding sequence is amplified by extending the first and second primer.

15. The method of claim 14 wherein the first and second primers are extended in a polymerase chain reaction (PCR).

16. The method of claim 1 wherein the sequence of the probe comprises at least 15 contiguous nucleotides complementary to the non-coding sequence.

17. The method of claim 1 wherein the sequence of the probe is substantially complementary to the entire non-

coding sequence between the coding sequence of the first and second histone genes of an organism of the sample.

18. The method of claim 1 wherein the eukaryotic organism is a species of protozoans, algae, cnidarians, annelids, nematodes, ciliates, yeast and mycelial fungi.

19. The method of claim 18 wherein the species of mycelial fungi or yeast is selected from the group consisting of *Alternaria alternata, Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus versicolor, Blastomyces dermatiditis, Candida albicans, Candida dubliniensis, Candida krusei, Candida parapsilosis, Candida tropicalis, Candida glabrata, Coccidioides immitis, Cryptococcus neoformans, Epidermophyton floccosum, Histoplasma capsulatum, Malassezia furfur, Microsporum canis, Mucor spp., Paracoccidioides brasiliensis, Penicillium marneffei, Pityrosporum ovale, Pneumocystis carinii, Sporothrix schenkii, Trichophyton rubrum, Trichophyton interdigitale, Trichosporon beigelii, Brettanomyces clausenii, Brettanomyces custerii, Brettanomyces anomalous, Brettanomyces naardenensis, Candida himilis, Candida lusitaniae, Candida multigemmis, Candida utilis, Candida intermedia, Candida norvegensis, Candida saki, Candida solani, Candida tropicalus, Candida versatilis, Candida bechii, Candida famata, Candida lipolytica, Candida stellata, Candida vini, Debaromyces hansenii, Dekkera intermedia, Dekkera anomala, Dekkera bruxellensis, Endomyces fibuliger, Geotrichium candidum, Hansenula fabiani, Hanseniaspora uvarum, Hansenula anomala, Hanseniaspora guillermondii Hanseniaspora vinae, Hypopichia burtonii, Kluyveromyces lactis, Kloeckera apiculata, Kluyveromyces marxianus, Kluyveromyces fragilis, Leucosproidium scottii, Metschnikowia pulcherrima, Pichia anomala, Pichia guilliermodii, Pichia orientalis, Pichia fermentans, Pichia memranefaciens,*

Rhodotorula glutinis, Rhodotorula mucilaginosa, Rhodotorula rubra, Saccharomyces bayanus, Saccharomyces cerevisiae, Saccharomyces dairiensis Saccharomyces exigus, Saccharomyces uinsporus, Saccharomyces uvarum, Saccharomyces oleaginosus, Saccharomyces boulardii, Saccharomycodies ludwigii, Schizosaccharomyces pombe, Torulaspora delbrueckii, Torulopsis holmii, Torulopsis stellata, Yarrowia lipolytica, Zygoaccharomyces baili, Zygosaccharomyces bisporus and *Zygosaccharomyces rouxii.*

20. The method of claim 1 wherein the first primer is complementary to the first strand of the coding sequence of the first histone gene of a plurality of species of eukaryotic organisms.

21. The method of claim 2 wherein the second primer is complementary to the second strand of the coding sequence of the second histone gene of a plurality of species of eukaryotic organisms.

22. The method of claim 1 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H2a protein.

23. The method of claim 22 wherein the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence VGAGAPVYLTAVLEY (SEQ ID NO. 1 or GNVTIAQGGVLPN (SEQ ID NO. 3).

24. The method of claim 23 wherein the amino acid sequence is selected from the group consisting of GAPVYLT (SEQ ID NO. 2), QGGVVPN (SEQ ID NO. 4) and APVYLTAAV (SEQ ID NO. 5).

25. The method of claim 1 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H2b protein.

26. The method of claim 25 wherein the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence VLKQTHPDTG (SEQ ID NO. 6) or QTAVRLILPGELAKH (SEQ ID NO. 8).

27. The method of claim 26 wherein the amino acid sequence is QTHPDTG (SEQ ID NO. 7) or PGELAKH (SEQ ID NO. 9).

28. The method of claim 1 wherein the first primer is complementary to the first strand of the coding sequence which encodes the H3 protein.

29. The method of claim 28 wherein the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of MARTKQTA (SEQ ID NO. 10), PGTVALRE (SEQ ID NO. 11), ALREIRRYQ (SEQ ID NO. 12), GGVKKPHRY (SEQ ID NO. 13) and GGKAPRKQ (SEQ ID NO. 14).

30. The method of claim 1 wherein the first primer is complementary to the first strand of the coding sequence which encodes the H4 protein.

31. The method of claim 30 wherein the first primer is complementary to the first strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of GITKPAIRR (SEQ ID NO. 15), GKGGKGLGKGG (SEQ ID NO. 16), GKGGAKRHR (SEQ ID NO. 17), MSGGKSGGK (SEQ ID NO. 18) and QGITKPAIRR (SEQ ID NO. 19).

32. The method of claim 2 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H2a protein and the second primer is complementary to the second strand of the coding sequence that encodes the H2b protein.

33. The method of claim 2 wherein the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence VLKQTHPDTG (SEQ ID NO. 6) or QTAVRLILPGELAKH (SEQ ID NO. 8).

34. The method of claim 33 wherein the amino acid sequence is QTHPDTG (SEQ ID NO. 7) or PGELAKH (SEQ ID NO. 9).

35. The method of claim 2 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H2b protein and the second primer is complementary to the second strand of the coding sequence that encodes the H2a protein.

36. The method of claim 35 wherein the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence VGAGAPVYLTAVLEY (SEQ ID NO. 1 or GNVTIAQGGVLPN (SEQ ID NO. 3).

37. The method of claim 36 wherein the amino acid sequence is selectd from the group consisting of GAPVYLT (SEQ ID NO. 2), QGGVVPN (SEQ ID NO. 4) and APVYLTAAV (SEQ ID NO. 5).

38. The method of claim 2 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H3 protein and the second primer is complementary to the second strand of the coding sequence that encodes the H4 protein.

39. The method of claim 38 wherein the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of GITKPAIRR (SEQ ID NO. 15), GKGGKGLGKGG (SEQ ID NO. 16), GKGGAKRHR (SEQ ID NO. 17), MSGGKSGGK (SEQ

ID NO. 18) and QGITKPAIRR (SEQ ID NO. 19).

40. The method of claim 2 wherein the first primer is complementary to the first strand of the coding sequence that encodes the H4 protein and the second primer is complementary to the second strand of the coding sequence that encodes the H3 protein.

41. The method of claim 40 wherein the second primer is complementary to the second strand of the coding sequence that encodes the amino acid sequence selected from the group consisting of MARTKQTA (SEQ ID NO. 10), PGTVALRE (SEQ ID NO. 11), ALREIRRYQ (SEQ ID NO. 12), GGVKKPHRY (SEQ ID NO. 13) and GGKAPRKQ (SEQ ID NO. 14).

42. The method of claim 1 or 2 wherein the sequence of the first and/or second primer is degenerate

43. The method of claim 1 or 2 wherein the sequence of the first and/or second primer is non-degenerate.

44. The method of claim 1 wherein the reference nucleic acid is a panel of reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species.

45. The method of claim 43 wherein the panel of reference nucleic acids is immobilised on a solid support.

46. The method of claim 1 wherein the one or more reference nucleic acid molecules is a first reference primer complementary to a first strand of the non-coding sequence of one or more eukaryotic species and the method further comprises the steps of:

(i) extending the first reference primer that is hybridised to the first strand of the non-coding sequence to amplify at least a segment of said non-coding sequence;

and

(ii) detecting the amplified segment to thereby detect whether the eukaryotic species is present in sample.

47. The method of claim 46 further comprising the step of providing a second reference primer complementary to the sequence of the second strand of said non-coding sequence.

48. A kit for detecting the presence of one or more species of eukaryotic organisms, comprising:

(i) at least a first primer complimentary to the sequence of the first strand of the coding sequence of a first histone gene for amplifying at least a segment of the non-coding sequence to produce a probe substantially complementary to the non-coding sequence between the coding sequence of the first and second histone gene, and

(ii) instructions for use.

49. The kit of claim 48 further comprising a second primer complementary to the sequence of the second strand of the coding sequence of the second histone gene.

50. The kit of claim 48 further comprising one or more reference nucleic acid sequences comprising the non-coding sequences of one or more eukaryotic species.

51. A nucleic acid molecule produced by a method according to any one of claims 1 to 47.

52. A probe for identifying eukaryotic species comprising a nucleic acid molecule according to claim 51, together with a detectable moiety.

53. A primer for producing a nucleic acid molecule according to claim 51 or 52.

54. A kit for detecting a eukaryotic species, comprising a nucleic acid molecule according to claim 51 or a probe according to claim 52.

55. A kit for detecting a eukaryotic species comprising a probe according to claim 52.

56. A kit for detecting a eukaryotic species comprising a primer according to claim 53.

57. A method for detecting a eukaryotic species in a sample comprising the steps of:

(i) extracting one or more nucleic acid molecules from the sample, wherein the nucleic acid molecules comprise the non-coding sequence located between and contiguous with the coding sequence of a pair of divergently transcribed histone genes; and

(ii) hybridising the non-coding sequence from the sample with the non-coding sequence of one or more reference nucleic acids thereby detecting the eukaryotic species.

58. The method of claim 57 wherein the reference nucleic acid is a first reference primer complementary to a first strand of the non-coding sequence of one or more eukaryotic species and the method comprises the further steps of:

(iii) extending the first reference primer that is hybridised to the first strand of the non-coding sequence to amplify at least a segment of said non-coding sequence; and

(iv) detecting the amplified segment thereby detecting whether species is present in sample.

59. The method of claim 58 further comprising the step of contacting a second reference primer complementary to the sequence of the second strand of said non-coding sequence.

60. A method for detecting a eukaryotic species in a clinical sample comprising the steps of:

(a) contacting a first primer complementary to the coding sequence of a H3 histone gene located in said sample and a second primer complementary to the coding sequence of a H4 histone gene located in said sample and amplifying at least a segment of a non-coding sequence located between and contiguous with the coding sequence of the H3 and H4 histone genes to produce a probe substantially complementary to the non-coding sequence;

(b) comparing the probe with one or more reference nucleic acid molecules comprising non-coding sequences of one or more eukaryotic species thereby detecting whether the species is present in the clinical sample.

61. A kit for detecting a eukaryotic species according to a method according to claim 57 to 60.

FIGURE 1

Z.R small> ACC CTT TCT CAA AAG TCT GTG AAC TCT ACC AAC TGG GAA
S.c large> ACC TCT TCT TAG CAA TCT GTG CAC TCT ACC GAC TGG GAA
T.d > ACC TTT TCT TAA CAG TCT GTG AAC TCT ACC GAC TGG GAA
S.c small> ACC TCT TCT TAG CAA TCT GTG CAC TCT ACC AAC TGG GAA

Z.R small> GGT CAA ACC TGC CTT AGC AGA TCT TGA TTG AGA AGC CTT
S.c large> TGT CAA ACC AGC CTT AGC AGA TCT AGA TTG AGA AGC TTT
T.d > AGT TAG ACC AGC CTT AGC TGA TCT GGA TTG AGA AGC CTT
S.c small> TGT TAA ACC AGC TTT AGC AGA TCT AGA TTG AGA AGC TTT

Z.R small> AGC TGC GGA ACC TGC TTT ACC ACC TTT ACC ACC GGA **CAT**
S.c large> AGC AGC TGA ACC AGC TTT ACC ACC TTT ACC ACC GGA **CAT**
T.d > TGC GGC AGA ACC TGC TTT ACC ACC TTT ACC ACC TGA **CAT**
S.c small> AGC AGC TGA ACC AGC TTT ACC ACC TTT ACC ACC GGA **CAT**

Z.R small> TGTTATATTTGTTGTATTTGTGATTGTTGTTTGTGTTGTAGAAAAGTAAGA
S.c large> TTTATATTTTATATGTATGAAATTTGTTTGTTTTGAAGTTGTTTATTCACT
T.d > GTATTCTGTTTGTGTTGTGTGAGTTGAGAAGGAGATGTTGCCTCTGGATAG
S.c small> TATATATTAAATTTGCTCTTGTTCTGTACTTTCCTAATTCTTATGTAAAAA

Z.R small> GTAAAGAAATCATGTATCTCACCAGTAACCTTCCTCCTTTTATACCGTTAC
S.c large> GAGAAATAACCAAATCCGTATGATGATGTAGTATCAAGAAGAGAAGTACAG
T.d > ATTATAAGGCCCGTTTATATACGCTTCGTCCTTGACTTTACAAGACTAGGC
S.c small> GACAAGAATTTATGATACTATTTAATAACAAAAAACTACCTAAGAAAAGCA

Z.R small> CTTTCCCTTAGGAATTCGCCACTGACTGAATCCCCTGTTCCATTTTTTTCT
S.c large> ATTGGAAGTAAATAGATGATGGTTCAACAAGACCAGAAAATCTACAAGCTG
T.d > CTTTTGGCGCGCCCCCCTTGGACCAGAACCTTCACCGTGTGCTGTGAAAAA
S.c small> TCATGCAGTCGAAATTGAAATCGAAAAGTAAAACTTTAACGGAACATGTTT

Z.R small> CACCCGGTGTGTGTGAAATAACTGGAACGTTGGAATTCGGTTCCAAGAACT
S.c large> ATTAGGAGTCTGTCTTATTTATATATTTTTTAGGTCAAGACTTATTGCTAG

```
T.d        >  CCTGGAACGTTGAAGTCGGTTTCTTTCCGATCGCGACGAGTTCATTTTTCA
S.c small>  GAAATTCTAAGAAAGCATACATCTTCATCCCTTATATATAGAGTTATGTTT

Z.R small>  ATTAAAAGTGACCGTAGTTGATGAAAAATTAATTTTCTCTTAACTATAGGT
S.c large>  TATTTACGATCCACTGGCTGGCTTCGTGAACGGGGAAGGGGGTGAGAAAAG
T.d        >  AACAAAAAATTGATTTCGCTTAACTATGGATAGACAGCGCGATTGCCAAAT
S.c small>  GATATTAGTAGTCATGTTGTAATCTCTGGCCTAAGTATACGTAACGAAAAT

Z.R small>  ATCGGTCCGTACTACCGCATTCGGTAACTCCATTCTGAAAAATTCACACCC
S.c large>  ATTTTGAAATCAACAAAGTGGGCAATAACAAATAACAGCATGAGAAACCAC
T.d        >  GCGGAATCGTTCCGTGCTGAAAATTTCGCCTTCAGTTTATACATAGTTAAG
S.c small>  GGTAGCACGTCGCGTTTATGGCCCCCAGGTTAATGTGTTCTCTGAAATTCG

Z.R small>  AGGGGCGAAAAAATAGGTACGGTATTTAATTTAACAAAGGATTCCAAAAGG
S.c large>  ATATCTCTACGGGCGTTTCTTCAACAACGACGAGTTAACTATTGTGCTCTT
T.d        >  ATAGGTTTATTAGCGAAGTTATGAGAACACATCTTATGACTGCCCATAGTT
S.c small>  CATCACTTTGAGAAATAATGGGAACACCTTACGCGTGAGCTGTGCCCACCG

Z.R small>  TATAAAAAGAGACAAGGTATTGGTATAGAACACGATAATCGATCCTTACAA
S.c large>  TTTTTGAGCCACCAAATACACTCCATTCCAATAGCTTCGCACAGTGAGGCG
T.d        >  AAGCATTAATTGACTTGATCTTGAAGTGATTGAACTATTTCATATAAAAGG
S.c small>  CTTCGCCTAATAAAGCGGTGTTCTCAAAATTTCTCCCCGTTTTCAGGATCA

Z.R small>  CTACCCACCCGGTGATCCATTANATTCAAATCAAACACAACACATACAAAT
S.c large>  AAAATTTTGGAACAGCGCTAATGAATTATTTGTGAGCTCGGCGAGTTCAAA
T.d        >  AGGCCTTATTGGAGGTATTGAGAGTTTCTTAATTTTTCTAAATAAAAAATA
S.c small>  CGAGCGCCATCTAGTTCTGGTAAAATCGCGCTTACAAGAACAAAGAAAAGA

Z.R small>  CAAA
S.c large>  TTTGAAGAAAACGCGGTTGGGTCGTTAACTATGGTTAGACGCTCAATGTCG
T.d        >  CTAAACAAATCAANACAAACTAAANCNAATA
S.c small>  AACATCGCGTAATGCAACAGTGAGACACTTGCCGTCATATATAAGGTTTTG

Z.R small>
```

S.c large> CCCGAAAGGGAAGGCTGTTCTCACTTTTTCGCGCGTTGCACCCTTTCTTCC
T.d >
S.c small> GATCAGTAACCGTTATTTGAGCATAACACAGGTTTTTAAATATATTATTAT

Z.R small>
S.c large> GCGAAAAAAATGAGAACGATGGATTTAAAATCAAGAGAATTGGCCTTAGTAG
T.d >
S.c small> ATATCATGGTATATGTGTAAAATTTTTTTGCTGACTGGTTTTGTTTATTTA

Z.R small>
S.c large> TGGCAAATACTACCTTGGTTGGTTATCTTGTAACGATTGGTAAGAAAGGGG
T.d >
S.c small> TTTAGCTTTTTAAAAATTTTACTTTCTTCTTGTTAATTTTTTCTGATTGCT

Z.R small>
S.c large> CATCTCTGTTTTCTTGATGTATATAAACAACATGATTTGATCATCTCAGAT
T.d >
S.c small> CTATACTCAAACCAACAACAACTTACTCTACAACTA

Z.R small>
S.c large> GGTCAGATTTATTAAAGACGTTTCTCTTTCCGCATTTTCGATTATTGTTAT
T.d >
S.c small>

Z.R small>
S.c large> ATTAAATTTATCCTATATAGACAAGTCAAACCACAAATAAACCATACACACATACA
T.d >
S.c small>

Z.R small> **ATG** TCT GCT AAA GCT AAA AGA AAC CCG CTT CCA AGG CCC CAG
S.c large> **ATG** TCT GCT AAA GCC GAA AAG AAA CCA GCC TCC AAA GCC CCA
T.d > **ATG** TCT GCC AAA GCT GAA AAG AAA CCT GCT TCC NAA GCC CCA
S.c small> **ATG** TCC TCT GCC GCC GAA AAG AAA CCA GCT TCC AAA GCT CCA

Z.R small> CTG AAA AGA AGC CAG CTG CCA AGA AAA CCG CAT CTT CTG TCG
S.c large> GCT GAA AAG AAA CCA GCC GCT AAA AAG ACT TCC ACT TCC ACT

```
T.d        >  GCT GAG AAG AAA CCA GCT GCT AAG AAG ACT GCT TCT TCC GTT
S.c small>    GCT GAA AAG AAG CCA GCT GCC AAG AAA ACA TCA ACC TCC GTC

Z.R small>    AAG GTA AGG GTA AGA AGA ACA AGG CAA GAA AGG AGA CCT ACT
S.c large>    GAT GGT     AAG AAG AGA AGC AAG GCT AGA AAG GAA ACA TAC
T.d        >  GNC AGT NAC AAG AAG AGA AGT AAG GTC AGA AAG GAA ACT TAT
S.c small>    GAT GGT     AAG AAG AGA TCT AAG GTT AGA AAG GAG ACC TAT
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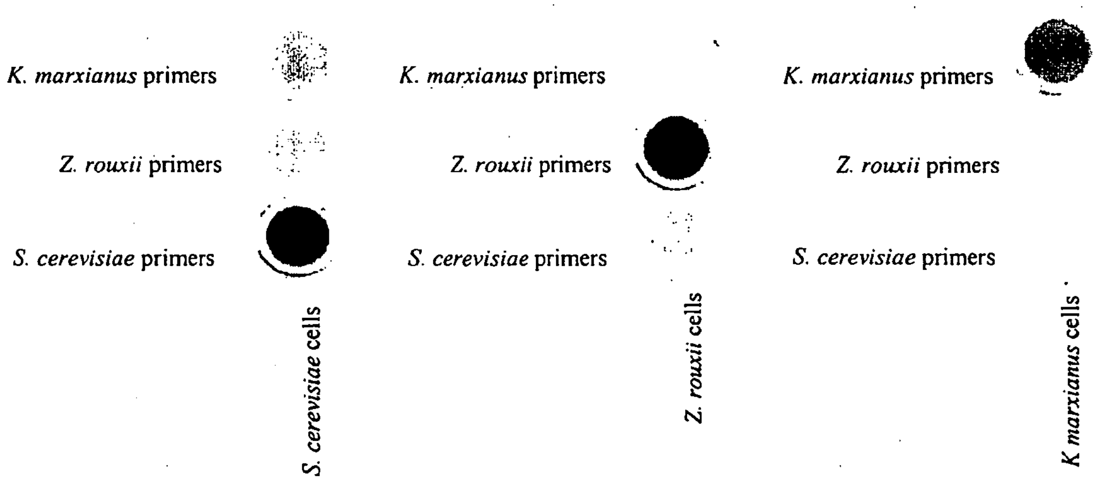
FIGURE 2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Z. rouxii primers
S. cerevisiae primers
K. marxianus primers
Z. rouxii primers
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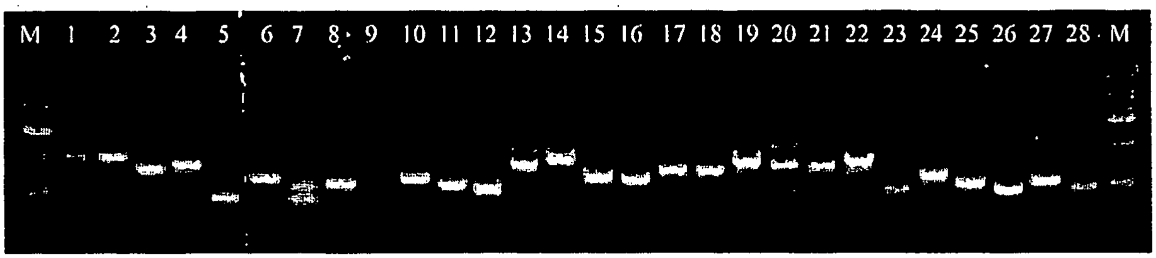
Z. rouxii primers
S. cerevisiae primers
S. cerevisiae cells
Z. rouxii cells
K marxianus cells

FIGURE 3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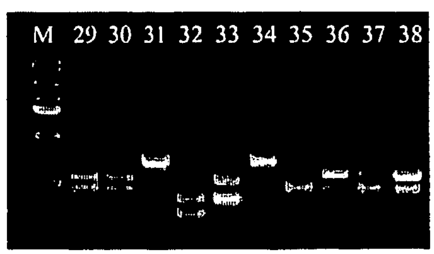
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FIGURE 4

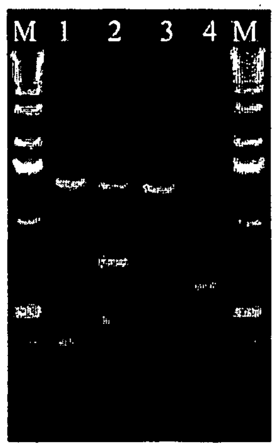

FIGURE 5

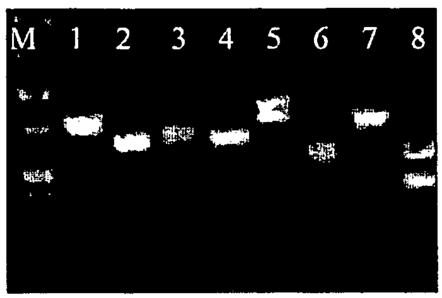

FIGURE 6

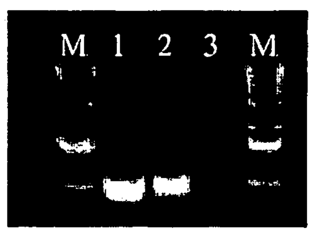

FIGURE 7

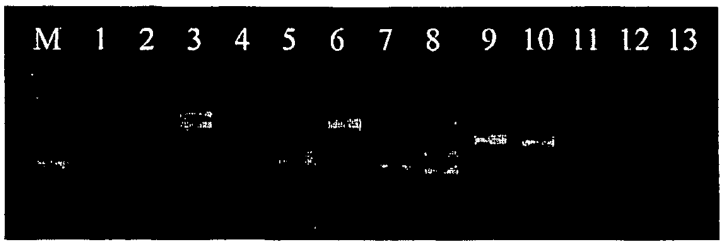

FIGURE 8

H2A1-H2B1 promoter region (Chromosome IV)

TGTATGTGTGTATGGTTTATTTGTGGTTTGACTTGTCTATATAGGATAAATTTAATATA
ACAATAATCGAAAATGCGGAAAGAGAAACGTCTTTAATAAATCTGACCATCTGAGATGA
TCAAATCATGTTGTTTATATACATCAAGAGAACAGAGATGCCCCTTTCTTACCAATCGT
TACAAGATAACCAACCAAGGTAGTATTTGCCACTACTAAGGCCAATTCTCTTGATTTTA
AATCCATCGTTCTCATTTTTTCGCGGAAGAAAGGGTGCAACGCGCGAAAAAGTGAGAAC
AGCCTTCCCTTTCGGGCGACATTGAGCGTCTAACCATAGTTAACGACCCAACCGCGTTT
TCTTCAAATTTGAACTCGCCGAGCTCACAAATAATTCATTAGCGCTGTTCCAAAATTTT
CGCCTCACTGTGCGAAGCTATTGGAATGGAGTGTATTTGGTGGCTCAAAAAAAGAGCAC
AATAGTTAACTCGTCGTTGTTGAAGAAACGCCCGTAGAGATATGTGGTTTCTCATGCTG
TTATTTGTTATTGCCCACTTTGTTGATTTCAAAATCTTTTCTCACCCCCTTCCCCGTTC
ACGAAGCCAGCCAGTGGATCGTAAATACTAGCAATAAGTCTTGACCTAAAAAATATATA
AATAAGACTCCTAATCAGCTTGTAGATTTTCTGGTCTTGTTGAACCATCATCTATTTAC
TTCCAATCTGTACTTCTCTTCTTGATACTACATCATCATACGGATTTGGTTATTTCTCA
GTGAATAAACAACTTCAAAACAAACAAATTTCATACATATAAAATATAAA

H2A2-H2B2 promoter region (Chromosome II)

TATTTGTAGAGTAAGTTGTTGTTGGTTTGAGTATAGAGCAATCAGAAAAATTTAACAAG
AAAGTAAAATTTTTAAAAAGCTAAATAAATAAACAAAATTAGTCAGTAAAAAAATTTTA
CACATATACCATGATATATAATAATATATTTAAAAACCTGTGTTATGCTCAAATAACAG
TTACTGATCCAAAACCTTATATATGACGGCAAGTGTCTCACTGTTGCATTACGCGTTGT
TTCTTTTCTTTGTTCTTGTAAGCGCGATTTTACCAGAACTAGATGGCGCTCGTGATCCT
GAAAACGGGGAGAAATTTTGAGAACACCGCTTTATTAAGCGAAGCGGTGGGCACAGCTC
ACGCGTAAGGTGTTCCCATTATTTCTCAAAGTGATGCGAATTTCAGAGAACACATTAAC
CTGGGGGCCATAAACGCGACGTGCTACCATTTTCGTTACCGTATACTTAGGCCAGAGAT
TACAACATGACTACTAATATCAAACATAACTCTATATATAAGGGATGAAGATGTATGCT
TTCTTAGAATTTCAAACATGTTCCGTTAAAGTTTTACTTTTCGATTTCAATTTCGACTG
CATGATGCTTTTCTTAGGTAGTTTTTTGTTATTAAATAGTATCATAAATTCTTGCCTTT
TACATAAGAATTAGGAAAGTACAGAACAAGAGCAAATTTAATATATA

H3-H4 promoter region (chromosome XIV)

TATTTTATTGTATTGATTGTTGTTTTTGCTACTCTTTTGAACAAGATGTAGAAAAAAAG
ATAGAGAAAAGAATAAATTAAGCGAAAAAAAAAAAATCTCTTTCACCGCCTCATCCTAAT
ATACTTATATATGATATAACTACATACGCACAAACACGTATGTATCTAGCCGAATAACA
ACAGCCCAGGCGCGAGTGAACAACATATTAAATTAAACGCCTTCTTGTCAGTTGTTTTG
TTCTGGTCTGGTCTGCATTTCGCGCCCGAAAAAGCTTGAGACGCGAAGCTCCCAGAACG
TCCTGCCATACAAATGCGAAACTCTCGGTCTAGTACCACTTTCCCGGCGCCAAACGACC
ACAGTTGTCCGTTCCGAGCACTTCGCATTAAGCGCGTGAAACTATTGGCACGCCATAAG
GGGTTCCTACGGATGGGAGTTGGTCATTTAGCGTTTATTATCGCCCAATGTGACGCACA
ATCACGGCTATGGCTCGGTGTCAAAACATAGTTTGCGTGATAACAGCGTGTTGTGCTCT
CTCGCGTTGCTTCTTGTGACCGCAGTTGTATATAAATAATCTTTTTCTTGTTCTTTTAT
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FIGURE 9a

FIGURE 9b

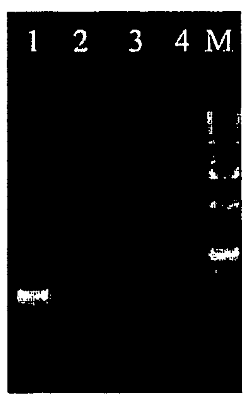

FIGURE 10a

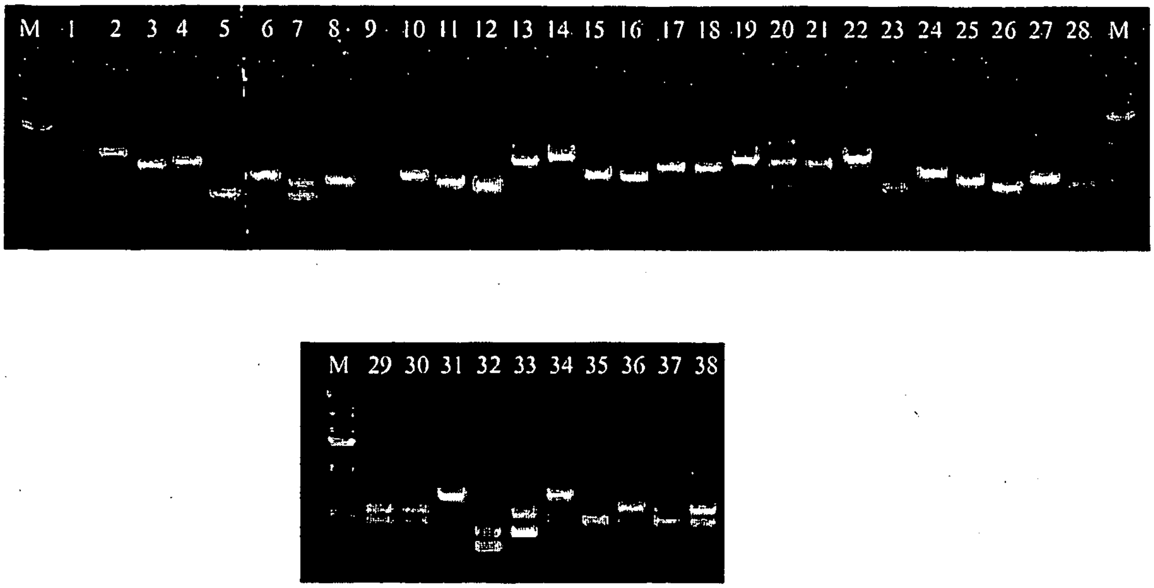

FIGURE 10b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00582

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int. Cl. [7]:	C12Q 1/68 C12N 15/11
According to International Patent Classification (IPC) or to both national classification and IPC	
B.	**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
WPIDS, CA

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, WPIDS, MEDLINE, BIOSIS (histone, promoter, intergenic, non-coding, bidirectional, diverg?, transcript?, transcrib?, phylogen?, identif?, diagnos?, fungus, fungi, yeast)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Baldo, A.M. *et al.* 1999. Potentials and limitations of histone repeat sequences for phylogenetic reconstruction of *Sophophora*. Molecular Biology and Evolution. 16(11):1511-1520. (See whole document, especially Figure 1 and pp1516-1517)	1-9, 12-27, 32-37, 42-59, 61
X	Sadler, L.A. and Brunk, C.F. 1992. Phylogenetic relationships and unusual diversity in histone H4 proteins within the *Tetrahymena pyriformis* complex. Molecular Biology and Evolution. 9(1):70-84. (See whole document)	1-7, 10-18, 20, 21, 28-31, 38-61

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 9 July 2003	Date of mailing of the international search report 14 JUL 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **DAVID OLDE** Telephone No : (02) 6283 2569

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sadler, L.A. and Brunk, C.F. 1990. Phylogenetic relations among *Tetrahymena* species determined by DNA sequence analysis. Molecular Evolution. Pp.245-252. (See whole document)	1-7, 10-21, 28-31, 38-61
X	Brunk, C.F. *et al.* 1990. Phylogenetic relationships among *Tetrahymena* species determined using the polymerase chain reaction. Journal of Molecular Evolution. 30(3):290-297. (See whole document)	1-7, 10-21, 28-31, 38-61
A	Van Den Bussche, R.A.*et al.* 2000. Characterization of histone H3/H4 gene region and phylogenetic affinity of *Ichthyophthirius multifiliis* based on H4 DNA sequence variation. Molecular Phylogenetics and Evolution. 14(3):461-468. (See whole document)	1-61
A	Bernhard, D. and Schlegel, M. 1998. Evolution of histone H4 and H3 genes in different ciliate lineages. Journal of Molecular Evolution. 46(3):344-354. (See whole document)	1-61
A	Marinets, A. *et al.* 1996. The sequence and organization of the core histone H3 and H4 genes in the early branching amitochondriate protist *Trichomonas vaginalis*. Journal of Molecular Evolution. 43(6):563-571. (See whole document)	1-61
A	Dobner, T. *et al.* 1991. A novel divergently transcribed human histone H2A/H2B gene pair. DNA Sequence. 1(6):409-413. (See whole document)	1-61
A	Sturm, R.A. *et al.* 1988. Conservation of histone H2A/H2B intergene regions: a role for the H2B specific element in divergent transcription. Nucleic Acids Research. 16(17):8571-8586. (See whole document)	1-61